Assessment and control of *Bacillus cereus* emetic toxin in food

Elina Jääskeläinen

Department of Applied Chemistry and Microbiology

Division Microbiology

University of Helsinki

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Supervisor: Prof. Mirja S. Salkinoja-Salonen
Department of Applied Chemistry and Microbiology
Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

Reviewers: Prof. Willem M. de Vos
1) Laboratory of Microbiology
Agrotechnology and Food Sciences Group
Wageningen University and Research Centre
Wageningen, the Netherlands
2) Department of Basic Veterinary Sciences
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Dr. Christophe Nguyen-The
Institute of Plant Products Technology
French National Institute for Agricultural Research (INRA)
University of Avignon
Avignon, France

Opponent: Prof. Jacques Mahillon
Laboratory of Food and Environmental Microbiology
Université Catholique de Louvain
Louvaine-la-Neuve, Belgium

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Front cover: Boys evaluating Mother`s art of cooking

To my family

Contents:

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IV. E.L. Jääskeläinen, M.M. Häggblom, M.A.Andersson, M.S. Salkinoja-Salonen. 2004. Atmospheric oxygen and other conditions affecting the production of cereulide by *Bacillus cereus* in food. International Journal of Food Microbiology 96: 75-83.

The author´s contribution

Paper I. Elina Jääskeläinen was responsible for the experimental work on LC-MS and wrote the article together with the other authors.

Paper II. Elina Jääskeläinen wrote the article and is the corresponding author. She also planned and carried out the experimental work except for some of the pH assays and the a_w assays.

Paper III. Elina Jääskeläinen wrote the article and is the corresponding author. She also planned and carried out the experimental work except for the manual extraction of cereulide, some of the boar sperm cell assays, cells cultivation and the Paju cell assays.

Paper IV. Elina Jääskeläinen wrote the article and is the corresponding author. She also planned and carried out all the experimental work.

Abbreviations

a_w	Water activity
ATCC	American Type Culture Collection
bceT	Enterotoxin T (*Bacillus cereus*)
Caco-2 cells	Colon adenocarcinoma
Calu-3 cells	Lung adenocarcinoma
cfu	Colony-forming unit
CytK	Cytotoxin K
$D_{95°C}$	Decimal reduction time at 95°C
D-value	Decimal reduction time
BCET-RPLA	*Bacillus cereus* enterotoxin reversed passive latex agglutination
BHI	Brain heart infusion
FDA	Food and Drug Administration (USA)
ESI	Electrospray ionization
EU	European Union
HBL	Haemolytic enterotoxin *by B. cereus*
HeLa cells	Derived from cervical cancer cells
HPLC	High-pressure liquid chromatography
IDF	International Dairy Federation
ISO	International Organization for Standardization
JC-1	5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenz-imidazolocarbocyanine iodide
kb	Kilobasepairs
Leu	Leucine
LC-MS	Liquid chromatography-mass spectrometry
Log K_{OW}	Logarithm of the n-octanol -water partition coefficient
Mb	Megabasepairs
m/z	Mass-to-charge ratio
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
NK cells	Natural killer cells
NMKL	Nordic Committee on Food Analysis
MYP	Mannitol egg yolk polymyxin agar
NHE	Nonhaemolytic enterotoxin
Paju cells	Human neural cell line
PCR	Polymerase chain reaction
Thr	Threonine
TSA	Tryptone soy agar
TSB	Tryptone soy broth
s.l.	*Sensu lato*
s.s.	*Sensu stricto*
UV	Ultraviolet
Val	Valine
$\Delta\Psi_m$	Mitochondrial inner membrane transmembrane potential
O-val	2-hydroxyisovaleric acid
O-leu	2-hydroxyisocaproic adic

Abstract

Despite of improving levels of hygiene, the incidence of registered food borne diseases has been at the same level for many years: there were 40 to 90 epidemics in which 1000-9000 persons contracted food poisoning through food or drinking water in Finland. Until the year 2004 salmonella and campylobacter were the most common bacterial causes of food borne diseases, but in years 2005-2006 *Bacillus cereus* was the most common. Similar developement has been published i.e. in Germany already in the 1990´s. One reason for this can be cereulide, the emetic toxin of *Bacillus cereus*. *Bacillus cereus* is a common environmental bacterium that contaminates raw materials of food. Otherwise than salmonella and campylobacter, *Bacillus cereus* is a heat resistant bacterium, capable of surviving most cooking procedures due to the production of highly thermo resistant spores. The food involved has usually been heat treated and surviving spores are the source of the food poisoning. The heat treatment induces germination of the spore and the vegetative cells then produce toxins. This doctoral thesis research focuses on developing methods for assessing and eliminating risks to food safety by cereulide producing *Bacillus cereus*. The biochemistry and physiology of cereulide production was investigated and the results were targeted to offer tools for minimizing toxin risk in food during the production.

I developed methods for the extraction and quantitative analysis of cereulide directly from food. A prerequisite for that is knowledge of the chemical and physical properties of the toxin. Because cereulide is practically insoluble in water, I used organic solvents; methanol, ethanol and pentane for the extraction. For extraction of bakery products I used high temperature (100°C) and pressure (103.4 bars). An alternative for effective extraction is to flood the plain food with ethanol, followed by stationary equilibration at room temperature. I used this protocol for extracting cereulide from potato puree and penne. Using this extraction method it is also possible to extract cereulide from liquid food, like milk. These extraction methods are important improvement steps for studying of *Bacillus cereus* emetic food poisonings. Prior my work, cereulide extraction was done using water. As the result, the yield was poor and variable.

To investigate suspected food poisonings, it is important to show actual toxicity of the incriminated food. Many toxins, but not cereulide, inactivate during food processing like heating. The next step is to identify toxin by chemical methods. I developed with my colleague Maria Andersson a rapid assay for the detection of cereulide toxicity, within 5 to 15 minutes. By applying this test it is possible to rapidly detect which food is causing the food poisoning. The chemical identification of cereulide was achieved using mass spectrometry. I used cereulide specific molecular ions, m/z (± 0.3) 1153.8 ($M+H^+$), 1171.0 ($M+NH_4^+$), 1176.0 ($M+Na^+$) and 1191.7 ($M+K^+$) for reliable identification. I investigated foods to find out their amenability to accumulate cereulide. Cereulide was formed high amounts (0.3 to 5.5 $\mu g\ g^{-1}$ wet wt) when cereulide producing *B. cereus* strains were present in beans, rice, rice-pastry and meat-pastry, stored at non refrigerated temperatures (21-23°C). Rice and meat pastries are frequently consumed under conditions where no cooled storage is available e.g. picnics and outdoor events.

Bacillus cereus is a ubiquitous spore former and is therefore difficult to eliminate from foods. It is therefore important to know which conditions will affect the formation of cereulide in foods. My research showed that the cereulide content was strongly (10 to 1000 fold differences in toxin content) affected by the growth environment of the bacterium. Storage of foods under nitrogen atmosphere (> 99.5 %) prevented the production of cereulide. But when also carbon dioxide was present, minimizing the oxygen contant (< 1%) did not protect the food from formation of cereulide in preliminary experiments. Also food supplements affected cereulide production at least in the laboratory. Adding free amino acids, leucine and valine, stimulated cereulide production 10 to 20 fold. In peptide bonded form these amino acids are natural constituents in all proteins. Interestingly, adding peptide bonded leucine and valine had no significant effect on cereulide production. Free amino acids leucine and valine are approved food supplements and widely used as flawour modifiers in food technology. My research showed that these food supplements may increase food poisoning risk even though they are not toxic themselves.

1. Background

The incidence of foodborne disease has increased during recent years (Varnam and Evans, 1991), despite improvement in hygiene. Food- and waterborne diseases are a significant cause of morbidity and mortality throughout the world. The reasons for this increase may lie in recent trends in global food production and changes in food technology in the industrialized countries. Reporting and diagnostic methods have also developed. *Bacillus cereus* is an endospore-forming bacterial species and a common cause of food poisoning in many countries.

Bacillus cereus produces many types of toxins, two of which are most frequently associated with food poisonings: 1) the thermolabile enterotoxins that are destroyed when food is heated and 2) the emetic toxin, which is not inactivated by heating of food (Jay *et al.,* 2005; Granum, 2007). Thermolabile *B. cereus* enterotoxins contaminating the raw materials of food are likely to be detoxified by heat, but no method is known for detoxifying the emetic toxin, cereulide, in food.

2. Review of the literature

2.1 The *Bacillus cereus* group

The genus *Bacillus* is a heterogeneous group of Gram-positive, spore-forming rods that belongs to the low G+C (Guanine+Cytocine) phylum Firmicutes (Holt *et al*., 1994). *Bacillus* subgroup 1 *(Bacillus cereus sensu lato* group) comprises the species *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus thuringiensis, Bacillus pseudomycoides, Bacillus weihenstephanensis* (Granum 2002; Jensen *et al.,* 2003) and the novel pathogen *Bacillus neocereus* (van der Zwet *et al.,* 2000, not yet a validly described species) . A close genetic relation was observed between all *B. cereus* group members (Helgason *et al*., 2000). Several characteristics have been suggested for differentiation of the *B. cereus* group (Table 1). The main diagnostic features of *B. cereus sensu lato* are their ability to hydrolyze lecithin and an inability to ferment mannitol.

Table 1. Differentation of members of the traditional *B. cereus* group (modified from: Granum 2007)

Properties	*B. cereus*	*B. anthracis*	*B. thuringiensis*	*B. mycoides*	*B. pseudomycoides*	*B. weihenstephanensis*
Motility	+/-	-	+/-	-	-	+/-
Penicillin susceptibility	-	+	-	-	n.d	-
Mannitol fermentation	-	-	-	-	-	-
Crystalline parasporal inclus	-	-	+	-	-	-
Hemolysis	+	-	+	(+)	n.d	+

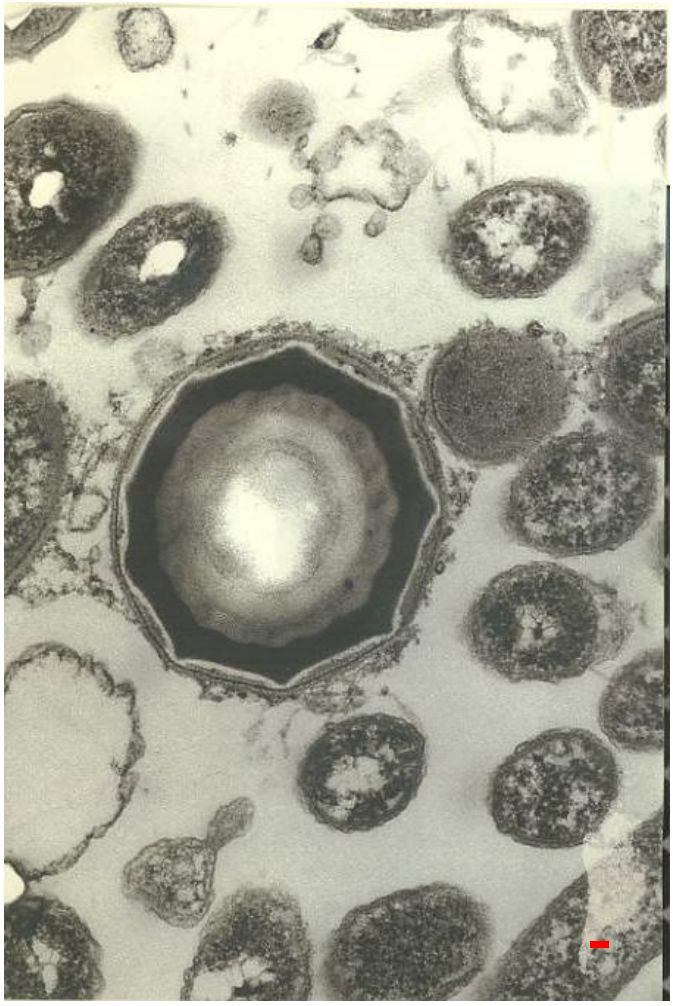
B. weihenstephanensis can be distinguished from *B. cereus* based on growth at < 7°C and not at 43 °C. *B. pseudomycoides* is not distinguishable from *B. mycoides* by physiological or morphological characteristics, but is differentiated based on fatty acid composition and 16S rRNA gene sequences. n.d. = not determined.

2.2 The species *Bacillus cereus*

2.2.1 Species description

Bacillus cereus was first isolated in 1887 from cowshed air by Frankland and Frankland (Roberts *et al*., 1996; Forsythe, 2000; Griffiths and Schraft, 2002). The cells under the microscope are rod-shaped, straight, typically 0.5-2.5 µm in diameter and 1.2-10 µm in length and are often arranged in pairs or chains (Holt *et al.,* 1994). The cells stain Gram-positive, although positive staining is often difficult to obtain in older cultures (Varnam and Evans, 1991). The cells are motile by peritrichous flagelli (Varnam and Evans, 1991). *B. cereus* endospores are centrally or pericentrally positioned. Figure 1 illustrates the spores of *B. cereus*. *B. cereus* is a mesophilic, facultatively anaerobic bacterium (Griffiths and Schraft, 2002) and is able to grow at redox-potential below -200 mV (Varnam and Evans 1991). *B. cereus* has an absolute requirement for three L- amino acids: threonine, leucine, valine (Agata *et al.,* 1999) as growth factors, but vitamins are not required (Griffiths and Schraft, 2002). The temperature range of growth is 4 - 55°C (optimum 30-40 °C) (Roberts *et al.,* 1996). Psychrotrophic strains are common and growth may occur at temperatures of 4-5 °C. The minimum water activity (a_w) for growth is 0.93 and the pH range is 4.3 - 9.3 (Forsythe, 2000).

Figure 1. Transmission electron micrograph of a sporulating culture of the emetic *B. cereus* strain F4810/72. The culture was grown for 10 days on Tryptic Soy Agar. Bar, 80 nm. Image taken by Maria Andersson.



2.2.2 The genome of *Bacillus cereus*

At least 12 *B. cereus* strains have been fully genome-sequenced by to date, (National Centre for Biotechnology Information NCBI, 2007). The published strains with finished sequence are listed in Table 2. The peptide synthase gene responsible for the nonribosomal synthesis of cereulide in emetic *B. cereus* strains (Ehling-Schulz *et al.*, 2005a) is extrachromosomal, located on an app. 200-kb plasmid (Hoton *et al*., 2005; Ehling-Schulz *et al.,* 2006; Rasko *et al.,* 2007). Even through the cereulide synthesis gene is located on a plasmid, the emetic strains that would produce both cereulide and the haemolytic diarrhoeal toxin, HBL, have not yet been reported (Guinebretière *et al*., 2002).

Table 2. The published complete genome sequences of *B. cereus* strains.

Bacillus cereus strain	Size (bp)	Number of genes	Links	Reference
ATCC10987	5224283	5603	NCBI, Refseq NC003909	Rasko *et al*., 2004
ATCC 14579	5411809	5234	NCBI, Refseq NC004722	Ivanova *et al*., 2003
ZK	5300915	5134	TIGR, Tax 288681	Han *et al*., 2006
NVH 391-98	4087024	4165	NCBI, Refseq NC009674	http://img.gi.doe.gov
E33L	5300915	5269	NCBI, Refseq NC006274	Han *et al.,* 2006

2.2.3 Detection and isolation of *Bacillus cereus*

The method for the enumeration of *B. cereus* in foods has been standardized by the International Organization for Standardization (ISO, 2004). The method is based on growth on mannitol egg yolk polymyxin (MYP) agar, in which the polymyxin B serves as a selective agent to suppress Gram-negative bacteria. The agar base contains D-mannitol as the fermentation substrate and phenol red as the indicator to detect formation of acid from mannitol. *B. cereus* cannot ferment mannitol, and thus no acid will be formed, while the colonies of *B. cereus* are pink due to the phenol red. The egg yolk produces a zone of precipitation around colonies with lecithinase activity, as is the case for most strains of *B. cereus sensu stricto.*

The lecithinase activity and negative reaction for mannitol fermentation are the most typical characteristics of *B. cereus* and also the basis for *B. cereus* identification according to Association of Official Analytical Chemists (AOAC, 1995), Nordic Committee on Food Analysis (NMKL, 1997), Food and Drug Administration (FDA, 1998) and International Dairy Federation (IDF, 1998). However, some *B. cereus* strains (mainly emetic), do not show the typical lecithinase reaction (Pirttijärvi *et al.,* 1999).

2.2.4 Spores of *Bacillus cereus*

All *Bacillus* species can form heat-stable endospores (for a recent review, see Henriques and Moran, 2007). *B. cereus* spores are an important factor in food-borne illness. The spores have a $D_{95°C}$ from below 1 min to over 30 min. The spores have no detectable metabolic activity and can survive in the absence of nutrients for many years. The first event in sporulation is an unequal division of the cytoplasm, resulting in large and small progeny each with the complete genome. After a series of morphological changes the mother cell lyses and releases the spore into the environment. There is no more than one spore per cell (Holt *et al.,* 1994). The process of spore formation requires about 6 h (Henriques and Moran, 2007).

An endospore is a dormant, tough and non -reproductive structure. The primary function of endospores is to ensure the survival of the bacterium through periods of environmental stress. The spores are highly resistant to heat, drying, toxic chemicals, UV radiation, gamma radiation and other adverse environmental factors. *Bacillus* spores are among the life forms most resistant to inactivation, with examples of spores being revived from amber 25-40 million years old or from brine inclusions dated at 250 -million years (Sagripanti *et al*., 2006; Henriques and Moran, 2007).

B. cereus sensu stricto spores have a more hydrophobic surface than any other *Bacillus* spp. spores. Therefore, they adhere to surfaces such as steel and plastics and are difficult to remove during cleaning (Granum 2002, 2007). Studies have revealed that the spores can adhere to Caco-2 cells in culture, indicating that they may adhere to the intestinal epithelium (Granum, 2007). The spores of some *B. cereus* strains are more resistant to heat than those of other mesophilic *Bacillus* spp. such as *B. subtilis* and *B. licheniformis* (Carlin *et al.,* 2006). *B. cereus* spores are capable of surviving most procedures applied in the cooking of food (Shinagawa *et al*., 1996). In collaboration with our laboratory, Carlin *et al.* (2006) investigated the heat tolerance of the spores of 17 cereulide-producing strains and 83 cereulide-nonproducing strains of *B. cereus* and reported that the spores of the emetic strains were many-fold more heat-resistant than those of the nonproducers. The spores of the strains producing emetic toxin exhibited higher D-values ($P < 0.001$) at 90 °C, as well as higher survival rates after 120 min of heating at 90 °C ($P < 0.001$), than did those of the non emetic strains (Carlin *et al*., 2006). These experimental facts show that emetic *B. cereus* strains in food are very difficult to destroy.

Since the spores are metabolically dormant, they must return to active growth, which they do through the process of germination. Germination consists of a series of degradative events, during which the various permeability barriers responsible for a significant degree of endospore resistance properties are broken down. These events result in rehydration of the core, facilitating entry of molecules from the external environment (Cronin and Wilkinson 2007; Henriques and Moran, 2007). The major germinant of *B. cereus* spores is inosine (Yousten, 1975; Hornstra *et al.,* 2007). Glycine and other neutral L-amino acids and purine ribosides induce germination (Warren and Gould, 1968; Griffiths and Schraft 2002; Hornstra *et al*., 2006). L-alanine is the most effective amino acid stimulating germination (Yousten 1975; Griffiths and Schraft 2002; Hornstra *et al*., 2007).

2.2.5 *Bacillus cereus* in the environment and in food

B. cereus is found in a wide range of habitats (Beattie and Williams, 2000), e.g. in soil and vegetation. The primary habitat of *B. cereus sensu lato* is most likely in the gut of arthropod invertebrates (Jensen *et al.,* 2003; Swiecicka and Mahillon, 2006), but it also colonizes the gut of small wild mammals including rodents and insectivores (Swiecicka and Mahillon, 2006). Since this bacterium is widespread in the environment, it enters the food chain through raw materials. It is a major problem in convenience foods and mass catering (Guinebretière *et al.,* 2006). The high resistance of the spores allows *B. cereus* to survive most drying and cooking processes. The organism grows well in cooked food because of the lack of a competing microbiota. *B. cereus* has been isolated from practically all nonsterile foods (Kolstø *et al.,* 2002; Granum, 2007). Few environments have been studied for the presence of cereulide producers. In the environments studied, only a minority of the *B. cereus* strains were cereulide producers (Table 3). In some foods, e.g. beans (Mikami *et al.,* 1994), cereulide-producing strains may be a substantial group.

Table 3. Reported frequencies of emetic *B. cereus* strains in the environment and in foods.

Environment	Country	Number of isolated *B. cereus* strains Total	Emetic	Method of cereulide detection	Reference
Infant foods	Finland	100	11	Boar sperm test, LC-MS	Shaheen *et al*., 2006
Dairy production chain	Sweden	5668	78	Boar sperm test, LC-MS	Svensson *et al*., 2006
Various foods (a)	NL	796	65	HEp-2 cell test	Wijnands *et al*., 2006
Various foods (b)	Japan	310	16	HEp-2 cell test	Mikami *et al.*, 1994
Ready-to-eat food	Denmark	40	1	PCR	Rosenquist *et al*., 2005
Soils and animal faeces	UK	101	0	MTT-test	Altayar and Sutherland 2005
Washed potato	UK	8	3	MTT-test	Altayar and Sutherland 2005
Food, human faeces, environments (c)	Japan	43	38	HEp-2 cell test	Nishikawa *et al*., 1996
Food, human faeces, environments (d)	Japan	76	4	HEp-2 cell test	Nishikawa *et al*., 1996
Pasta food (e)	Finland	122	83	Boar sperm test, LC-MS	Pirhonen *et al*., 2005

a) oils and fats and their products, fish and meat and their products, milk and milk products, pastry, vegetables and their products, ready-to-eat foods, flavourings
b) vegetables, fruits, grain, fermented foods
c) isolated from five emetic-syndrome outbreaks
d) isolates associated in other than emetic *B. cereus* food poisoning outbreaks
e) isolates associated in with an emetic-syndrome food-borne outbreak

2.3 *Bacillus cereus* food poisoning

In European legislation *B. cereus* is classified as a Hazard group 2 organism, based on its ability to cause infections in humans (European Commission, 1993). In addition, it is the causative agent of two distinct types of food poisonings. *B. cereus* was first proven to cause food-borne disease in 1950. The food was highly contaminated vanilla sauce and consumption resulted in a diarrhoeal illness (Jay *et al.,* 2005). About 20 years later *B. cereus* was also recognized to cause an emetic type of gastrointestinal disease; in 1971 many cases associated with *B.cereus* in fried rice from Chinese restaurants (Mortimer and McCann, 1974). Subsequently, *B. cereus* was recognized as an important cause of food poisoning worldwide.

In 2005 a total of 55 food poisoning outbreaks, in which food or drinking water was shown to be the causative agent, were registered in Finland (Niskanen *et al.,* 2006). The causative agent for the outbreaks remained unidentified in 19 outbreaks (38%). Five epidemics (10%, involving 64 persons) were caused by *B. cereus*, i.e. more than any other recognized bacterial agent in Finland. In year 2006 similar developement has continue (Niskanen *et al.,* 2007). *B. cereus* is also a major problem in convenience foods and mass catering in other European countries (Guinebretière *et al*; 2002, 2006; Wijnands *et al*., 2006). The *B. cereus* toxins that cause fatal poisonings in humans include cytotoxin K (Lund *et al.,* 2000) and cereulide (Mahler *et al*., 1997; Dierick *et al*., 2005). In addition, *B. cereus* may have been involved in outbreaks involving heated foods, in which no viable bacteria could be isolated. Cytotoxin K is a protein consisting of a single polypeptide chain (Fagerlund *et al.,* 2004). Cereulide, the emetic toxin of *B. cereus*, is a nonribosomally synthesized small peptide that can survive heating, but neither authorities nor food manufacturers analyse foods or raw materials routinely for cereulide. The reporting rate of illness caused by *B. cereus* may also be underestimated, due to the usually short duration (often < 24 h) of the diarrhoeal and emetic syndromes (Granum 2007). Consequently the full extent of *B. cereus* food poisoning in Finland, as well as in other countries, is yet unknown.

The reported food-borne outbreaks and cases attributed to *B. cereus* in North America, Europe and Japan range from 1% to 22% for outbreaks covering 0.7- 33% of the cases (Griffiths and Schraft 2002). The Netherlands and Norway were reported to have the most extensive problem due to *B. cereus* (Griffiths and Schraft 2002). The type of food-borne illness caused by *B. cereus* varies among countries. In Japan, the emetic syndrome is about 10 times more frequently reported than the diarrhoeal disease, while in Europe the diarrhoeal illness is more frequently reported. This difference is presumably due to the differences in food and cooking traditions among these areas (Granum, 2007).

2.3.1 Diarrhoeal food-borne infection by *B. cereus* and its causative agents

Diarrhoeal illness due to ingestion of *B. cereus* spores is characterized by abdominal pain and diarrhoea. The incubation period is 8-16 h and the symptoms persist for 12-24 h (Sim 1998; Beattie and Williams, 2000; Granum 2007). The diarrhoeal syndrome caused by *B. cereus* is mediated by one or the three diarrhoeal enterotoxins (Table 4): the tripartite toxins haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE), the two forms of cytotoxin K (cytK-1 and cytK-2) (Fagerlund *et al.,* 2004) and possibly enterotoxin T and enterotoxin FM (Guinebretière *et al.,* 2002; Moravek *et al.,* 2006) . The proteolytic enzymes and pH of the gastrointestinal tract digest these enterotoxins if they are preformed in foods. *B. cereus* spores survive digestion and may germinate in the intestine (Jensen *et al.,* 2003; Swiecicka *et al.,* 2006), while the vegetative cells may produce toxin in the gut.

Two immunological assays are commercially available for the detection of *B. cereus* diarrhoeal toxins. BCET-RPLA: *Bacillus cereus* enterotoxin reverse passive latex agglutination (Oxoid, Basingstoke, UK) detects the L_2 component of the haemolysin (Granum 2002). The Tecra (Batley, UK) Bacillus diarrhoeal enterotoxin visual immunoassay (BDE-VIA) detects the 45- kDA protein of the non-haemolytic enterotoxin (Lund and Granum, 1996). A number of cell lines are also susceptible to the diarrhoeal toxins, e.g. Vero (monkey kidney) and CHO (Chinese hamster ovary). The diarrhoeal enterotoxin is produced in the gut by germinating spores of *B. cereus* (Granum, 2007). Some *B. cereus* strains may stably colonize the gut of at least arthropods (Swiecicka and Mahillon, 2006). There is no documentation on the illness-causing effect of the toxins in ingested food. Most likely the

ingested toxin proteins are inactivated in the human digestive tract by proteolytic enzymes. The presence of diarrhoeal toxin genes can be detected by using polymerase chain reaction (PCR) (Guinebretière *et al.,* 2002, 2006; Abriouel *et al*., 2007; Fagerlund *et al.,* 2007). In food-borne isolates of *B. cereus*, the presence of a toxin gene does not prove that the bacterium actually produced the diarrhoeal toxin in the human gut. Since *B. cereus* in heated foods is always present as spores, the actual illness will only occur if the spores germinate in the gut. Wijnands *et al.* (2007) showed that germinants from differentiated Caco-2 cells induced spore germination in *B. cereus.*

Table 4. Enterotoxins known to be produced by *Bacillus cereus.* Modified from: Granum 2002.

Toxin	Type/ size	Food poisoning	Commercial detection method
Haemolysin BL	Protein, 3 components (46, 38, 37 kDa)	Probably	Oxoid assay
Nonhaemolytic Enterotoxin (NHE)	Protein, 3 components (41, 40, 36 kDa)	Yes	Tecra kit
Cytotoxin K1, K2	Protein, 1 components (34 kDa)	Yes	No
Enterotoxin T	Protein, 1 component (41 kDa)	Unknown	No
Enterotoxin FM	Protein, 1 components (45 kDa)	Unknown	No

2.3.2 Emetic food-borne intoxication

The causative agent of *B. cereus* emetic food poisoning is a ring-structured dodecadepsipeptide 1.2 kDa in size, first identified by Agata *et al.* (1994). Cereulide consists of only three repeats of 2 amino acids and 2 hydroxy acids: D-O-leu-D-Ala-L-O-Val-L-Val (Figure 2). Cereulide structurally resembles valinomycin produced by strains of *Streptomyces* (Agata *et al.,* 1994). Both cereulide and valinomycin are potassium ionophores (Mikkola *et al.,* 1999; Teplova *et al.,* 2006; Andersson *et al*., 2007). Cereulide is resistant to heat, extremes of pH and to the proteolytic activities of pepsin and trypsin (Kramer and Gilbert, 1989). If the ingested food contains cereulide, the toxin is likely to remain intact and will likely become sorbed from the gut in its active toxic form. The molecular properties of cereulide are listed in Table 5.

Together with the cytotoxin K (Lund *et al.,* 2000), cereulide is regarded as the most dangerous to human health of the toxins produced by *B. cereus*, because it is responsible for deaths of young healthy persons. A 17-year-old boy in Switzerland died of fulminant liver failure caused by mitochondrial damage after consuming food contaminated with *B. cereus* and its emetic toxin (Mahler *et al*., 1997). Similarly, a 7-year-old girl in Belgium died only 13 h after ingesting *B. cereus*- contaminated pasta salad (Dierick *et al*., 2005). The significance of cereulide has probably not been recognized in liver failures of unknown aetiology.

The toxin preformed in food may cause symptoms 0.5-5 h after ingestion of the contaminated food. The illness is characterized by nausea and vomiting lasting for 6-24 h. In the stomach, cereulide will interfere with 5-HT3 (serotonin) receptors of the nervus afferent that enervates the stomach. Dissecting this nerve resulted in loss of the emetic response to ingested cereulide in an insectivore, the house musk shrew (*Suncus murinus*) (Agata *et al*., 1995). Cereulide inhibits the cytotoxic activities and cytokine production of human natural killer cells and is thereby a potential immunosuppressant (Paananen *et al.*, 2002). The toxic properties of cereulide are listed in Table 6.

Extracts from the emetic strains of *B. cereus* induced emesis in rhesus monkeys *(Macaca mulatta*) and in musk shrews (*Suncus murinus)* (Table 5). Yokoyama *et al.* (1999) showed that mice were insensitive to orally given synthetic cereulide, but when cereulide was injected intraperitoneally it caused vacuolization and mitochondrial swelling in the liver, similar to that reported in a fatal human case (Mahler *et al.,* 1997). The hepatocytes showed mitochondrial swelling, with loss of cristae from the mitochondria, and dose-dependent increases in small fatty droplets in the cytoplasm. At higher doses of cereulide, massive degeneration of hepatocytes occurred in the mouse. In the mouse, regeneration of hepatocytes was observed 4 weeks after exposure to 10 µg of cereulide per mouse, at 25 µg of cereulide per mouse, the mice died within hours.

Emetic food poisoning is often associated with rice foods. Many incidents of emetic illness are associated with starchy foods such as mashed potatoes (Jay *et al.,* 2005). Griffiths and Schraft (2002) suggested that starch may promote the growth of *B. cereus* and the production of emetic toxin. Emetic *B. cereus* strains are mainly unable to hydrolyse starch (Shinagawa 1993; Agata *et al.* 1996; Pirttijärvi *et al.,* 1999, 2000) and starchy foods will look and taste

fine even though emetic *B. cereus* colony counts may be high. This may explain why emetic food poisonings are usually associated with starchy foods. The only studies so far, in which the contents of cereulide were estimated in more than one food implicated in emetic-type *B. cereus* food poisoning, were published by Agata *et al.* (1999, 2002). (Table 7). The cereulide levels of foods estimated in their study ranged from < 5 to 1280 ng/g. The emetic toxin content of the foods in their study was estimated, based on the toxicity titres of aqueous supernatants of foods measured using human larynx carcinoma (HEp-2) cells. Therefore the numbers are only accurate up to the dilution step. The exact toxin dose in humans is difficult to measure, even if a more accurate analysis is used, because the toxin is likely to be inhomogenously distributed in most foods.

Figure 2. Structure of cereulide. Picture from:
http://www.biocenter.helsinki.fi/groups/salkinoja/index.htm

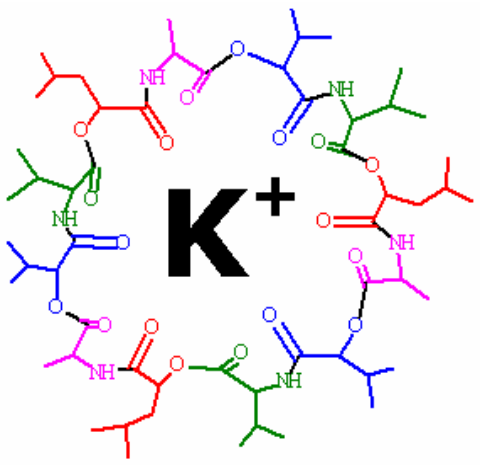


Table 5. Molecular properties of cereulide.

		Reference
Molecular structure	Cyclic dodecadepsipeptide, 1152g/mol	Agata *et al.*, 1994; Mikkola *et al*., 1999
Synthesis	Nonribosomal	Hoton *et al*. 2005; Ehling-Schulz *et al.*, 2006; Rasko *et al*., 2007
Sensory properties	Colourless, odourless	
Octanol-water coefficient	Log Kow 6.0	Teplova *et al*., 2006
Heat stability	No loss of activity upon cooking or autoclaving	Mikami *et al*., 1994; Shinagawa *et al*., 1996
pH stability	Stable between 2-11	Mikami *et al.*, 1994; Shinagawa *et al*., 1996
Black-lipid membrane	Potassium ionophore	Mikkola *et al*., 1999; Teplova *et al*., 2006; Andersson et al., 2007
Isolated rat liver mitochondria	Catalyses afflux of K+ ions	Teplova *et al.*, 2006
Mode of cell action	Depolarized mitochondrial membrane	
	of boar sperm cells	Hoornstra *et al*., 2003
	of NK cells	Paananen *et al.*, 2002
	of neural cells	Teplova *et al*., 2004
	of isolated rat liver mitochondria	Kawamura-Sato *et al*., 2005

Table 6. Food poisoning properties of cereulide, the emetic toxin of *B. cereus*.

		Reference
Emetic dose	10 µg toxin/kg rhesus monkey (*Macaca mulatta)*	Shinagawa *et al.*, 1995
	8 -10 µg toxin/kg house musk shrew (*Suncus murinus*)	Agata *et al.*, 1995
Incubation period	0.5-6 h	Beattie and Williams, 2000
Duration of illness	6-24 h	Griffiths and Schraft, 2002
Production	Preformed in food	Granum, 2007
Trypsin digestion	Not cleaved by trypsin	Mikami *et al.*, 1994, Shinagawa *et al*., 1996
Prevalence in foods	Many foods like, e.g. rice, pastries, pasta, noodles	Jay *et al*., 2005

Table 7. Emetic toxin contents in food samples implicated in *B. cereus* emetic-type food poisoning (Agata *et al.,* 2002). Similar foods and emetic toxin contents were published earlier by the same author (Agata *et al.,* 1999).The toxin contents were measured with by HEp-2 cell vacuolation activity of the centrifuged and then autoclaved supernatants of foods. A food homogenates were prepared in distilled water, using the stomacher instrument.

Food	Cereulide titer (ng/g)
Fried rice 1	1280
Fried rice 2	160
Fried rice 3	160
Fried rice 4	< 5
Boiled rice 1	640
Boiled rice 2	320
Boiled rice 3	160
Boiled rice 4	80
Boiled rice 5	10
Spaghetti 1	80
Spaghetti 2	40
Noodle	20
Curry and rice	80

2.3.3 Specific features of emetic toxin-producing strains of *B. cereus*

Shinagawa (1993), Agata *et al.* (1996) and Nishikawa *et al.* (1996) concluded, based on phenotypic properties, that emetic toxin production was associated with a specific class of *Bacillus*. This was later supported by analysis of chemotaxonomic and genotypic properties (Pirttijärvi *et al*., 1999; Ehling-Schulz *et al.,* 2005b). In the past decade, more information accumulated and it is now evident that cereulide-producing strains may be more diverse than previously believed (Apetroaie *et al*. 2005; Vassileva *et al*., 2007). Most currently described emetic strains of *B. cereus* share the originally described features, such as being negative for salicin fermentation and for starch hydrolysis (Shinagawa 1993; Agata *et al.* 1996), but exceptions are also being found. Thorsen *et al.* (2006) recently described two starch-positive cereulide-producing strains of *B. weihenstephanensis*. Most of the known cereulide-producing strains belonged to serovar H1. However, some emetic toxin-producing strains belong serotypes H3 and H12 (Taylor and Gilbert, 1975; Hughes *et al.,* 1988; Agata *et al.,* 1996, Vassileva *et al*., 2007).

The *B. cereus* group strains display an extremely large array of ribopatterns. A majority of the emetic toxin-producing strains, studied by the year 2000, possessed identical ribopatterns (Pirttijärvi *et al*., 1999; Ph. D thesis of Tuija Pirttijärvi 2000). Recently, emetic strains with novel ribopatterns were reported (Apetroaie *et al*., 2005; Shaheen *et al.*, 2006).

2.3.4 Methods for detecting and quantifying cereulide

Reagents or equipment for detecting and/or quantifying cereulide are not yet commercially available. The gold standard for emetic toxin detection is the monkey-feeding assay (Griffiths and Schraft, 2002). However, by European legislation (Registration, Evaluation, Authorization and Restriction of Chemicals, REACH), effective as of June 1st 2007, whole animals are not allowed for food testing. Consequently, *in vitro* assays must be used for toxin detection in food. Cereulide causes vacuolation of the mitochondria in HEp-2 (human larynx carcinoma) cells. This can be visualized under a light microscope (Hughes *et al*., 1988). Finlay *et al.* (1999) described a modified, more sensitive HEp-2 cell test. The method is based on the use of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), which detects the mitochondrial dehydrogenase, regarded as an indicator of cell viability. The detection limits of various cereulide assays are compiled in Table 8.

Our laboratory developed a test (Andersson *et al.,* 1998) based on boar spermatozoan motility. The plasma membrane of boar sperm cells has a low sterol content and is therefore highly permeable to hydrophobic molecules, such as cereulide (Table 5). The motility of boar spermatozoa is dependent on correct functioning of the mitochondria. Inhibited motility may be an indication of mitochondrial damage. Motility inhibition can be observed using a light microscope (Andersson *et al.,* 1998) or using a commercially available sperm analyser (Rajkovic *et al*., 2006b). The mitochondrial electric transmembrane potential ($\Delta\psi m$) of mammalian cells can be visualized by staining with JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide). The lipophilic fluorochrome JC-1 changes its emission spectrum, depending on the level of $\Delta\psi$ (Reers *et al.,* 1995). The first effect visible after exposure of sperm cells to cereulide was hyperpolarization of the plasma membrane, which occurred within 5 min of exposure to the bacterial extract. Subsequently, the sperm cells lost motility and the mitochondria became depolarized.

Cereulide easily dissolves in organic solvents and can thus be identified and quantitated by high-pressure liquid chromatography (HPLC) combined with mass spectrometry (MS). The first report for this accurate cereulide quantification method was published in 2002 (Häggblom *et al*., 2002). Separation was done, using HPLC and quantitative analysis by determining the concentration of the indicative mass ions specific for cereulide with an ion trap mass spectrometer. Cereulide and valinomycin have similar responses (same toxin concentration → same peak area in MS) in HPLC-MS analysis (Häggblom *et al.,* 2002). Since purified cereulide is not commercially available for use as a standard, valinomycin was used as a reference for quantification of cereulide.

PCR-based assays were recently developed for identifying potentially emetic *B. cereus* strains (Ehling-Schulz *et al*. 2004, Horwood *et al*., 2004). The PCR is a reliable method for detecting the presence of the cereulide synthase gene (Fricker *et al.,* 2007) . The presence of the toxin synthase gene is required for producing cereulide. However, its presence does not show whether the bacterium actually produces or if the food contains cereulide in concentrations sufficient to cause disease.

The actual production of cereulide is strain-dependent (Apetroaie *et al*., 2005) and also strongly affected by the environment (Shaheen *et al.,* 2006) . For assessing the risk of food poisoning by emetic *B. cereus,* direct analysis of this toxin in food is needed. Risk assessment should therefore be based on the toxin actually present in the food or the likelihood of toxin formation in food or in the gut.

Table 8. Lowest limit of various methods used for cereulide detection.

Method for detection	**Toxicity endpoint**	**Detection limit**	**Reference**
Toxicity titer based methods			
HEp-2 cells	Vacuolation	ca. 1-5 ng per ml	Mikami *et al.* , 1994
MTT method	Vacuolation, staining	ca. ≥ 0.5 ng per ml	Finlay *et al.,* 1999
Boar sperm cell test	Motility	0.5 ng per ml	Andersson *et al.,* 1998
Direct chemical analysis			
LC-MS		10 pg per injection	Häggblom *et al.,* 2002

2.4 Emetic toxin production by *B. cereus* in different growth environments

Various growth media have been evaluated as production media for the emetic toxin of *B. cereus*. Szabo *et al.* (1991) first reported that commercial skim milk is a good environment for the *B. cereus* heat-stable toxin production measured by the HEp-2 cell assay. Cereulide is so far the only known heat-stable *B. cereus* toxin found in foods. Therefore, Szabo *et al*. (1991), and many other authors (Table 6 and Agata *et al*., 1999, 2002) who used toxicity assays to measure the emetic *B. cereus* toxin most likely measured toxicity caused by cereulide. Molecular identification of cereulide was done later by Agata *et al*. (1994) and by Mikkola *et al*. (1999). Wang *et al.* (1995) described homocereulide with a molecular mass of 1166 Da. These authors isolated homocereulide from a marine *B. cereus* strain SCRC and showed its potent cytotoxicity. However, homocereulide has never been shown to act as the emetic toxin nor has it been associated with food poisonings.

Agata *et al.* (1999) reported that cereulide titres were higher in commercial skim milk media than in brain heart infusion broth (BHI), trypticase soy broth (TSB) or nutrient broth when these were preinoculated with the same strain of *B. cereus*, NC7401. These authors also developed a chemically defined medium for cereulide production (Agata *et al*., 1999). Three amino acids: L-valine, L-leucine and L-threonine are essential for *B. cereus* growth as well as for the production of cereulide.

Agata and coworkers (2002) studied cereulide production in different foods. Various consumer foods were seeded with *B. cereus* strain NC7401, added in amounts of 10^3 cfu/g. After incubation for 24 h, they prepared the food as a suspension in distilled water, cleared the suspension by centrifugation and then measured in the supernatant the titer of heat-stable toxicity compared with those of other food tested. They obtained (based on toxicity) the highest cereulide contents (320 ng/g) in boiled rice. Water extracts from similarly treated bread and cake contained only 20 ng of cereulide per g of extracted food when the incubation time and temperature were the same. In egg and its products only low amounts, < 5-10 ng /g, of cereulide were extracted.

Szabo *et al.* (1991) also found that white rice, inoculated with *B. cereus* strain F4810/72, accumulated at 27 °C within 18 h more water-extractable heat-stable toxin (toxin titer 512)

than did brown rice (toxin titer 128) or converted rice (toxin titer 256). In the present study the toxicity assay was not calibrated, so the toxin titers cannot be compared with those in other works.

The cereulide productivity of *B. cereus* strains was reported to be sensitive to ambient temperatures (Häggblom *et al*., 2002). Szabo *et al.* (1991) reported the optimum temperature for emetic toxin production as 20 - 30 °C. Most emetic strains of *B. cereus* grow at temperatures of over 40 °C (Häggblom *et al*, 2002) and some up to 52 °C (Carlin *et al.,* 2006; Ehling-Schulz *et al*., 2006). Cereulide production by the strain F4810/72 was nondetectable at ≤ 8 °C and at 40 °C (Häggblom *et al.,* 2002).

Finlay *et al*. (2000) showed that low temperatures (10 °C) suppressed the growth of and thus also emetic toxin production by the *B. cereus* strains F4810/72, F3748/75, F3744/75, F4562/75, F4552/75, F2427/75 and F2549A , F5881, F4810/72, NS117 and NS115 in skim milk medium. Similarly, Häggblom *et al.* (2002) showed that cereulide production by *B. cereus* strains F4810772, NC7401 and F5581 was detectable, but low below 12 °C in tryptic soy broth. Rajkovic *et al*. (2006a), used brain hearth infusion broth as the medium and showed that *B. cereus* strains F4810/72, NS115 and NS117 produced no emetic toxin at 12 °C. Thorsen *et al.* (2006) reported that two strains of the psychrotolerant species, *B. weihenstephanensis*, may produce cereulide. These two strains grew at temperatures as low as 8 °C, but produced cereulide only at 25 °C.

Häggblom *et al.* (2002) reported that cereulide production by the *B. cereus* strains NC7401 and F4810/72 in stationary incubated Trypticase soy broth was undetectable (< 0.02 μg ml^{-1}) compared with cultures incubated on a rotary shaker at 150 rpm (> 1 μg ml^{-1}) during 24 h . Agata *et al*. (2002) and Finlay *et al.* (2002) observed 90% more emetic toxin production in shaken milk as than in stationary incubated milk. However, Shaheen *et al.* (2006) inoculated infant food formulas with the *B. cereus* strain F4810/72. They found little cereulide in dairy-based formulas, whether shaken or not, but found much higher cereulide concentrations (50 x) when cereal-based infant formula foods were stationary-incubated compared with moderate (60 rpm) shaking for 24 h at 21-23 °C.

Rajkovic *et al*. (2006a) studied cereulide production in laboratory media under atmospheres with differing oxygen contents. In their study the head space gas composition was controlled with a CO_2/O_2 gas analyser. These authors found that no cereulide accumulated in TSA plate-grown cultures (*B. cereus* emetic strains NS117 and 5964a) when the atmosphere contained less than 1.6 vol % O_2, but when the O_2 concentration was 4.5 vol %, high amounts (about 1000 ng mg^{-1}) of cereulide accumulated.

3. Aims of this study

This doctoral thesis research focuses on developing methods for assessing and eliminating risks to food safety by cereulide-producing *Bacillus cereus*. The biochemistry and physiology of cereulide production were investigated and the results targetted to offer tools for food production technology to minimize the toxin risk.

The specific goals were to:

1. Develop methods useful for rapid scoring of cereulide production among *B. cereus* isolates from foods or from the environment.

2. Develop methods for quantitative extraction and analysis of cereulide directly from food.

3. Identify conditions under which cereulide production by *B. cereus* may occur or not occur in selected growth media or foods.

4. Compare the mitochondrial toxic effects of cereulide in mammalian somatic cells and germ cells.

4. Materials and methods

The methods used in this study are listed in Table 9.

Table 9. Methods used in this study

Analysis	Description	Reference, manufacture
Extraction methods:		
Extraction of cereulide from bacterial cultures	Paper I	
Extraction of cereulide from food	Paper II	
Extraction of cereulide from liver	Chapter 5.6	
Assays for toxicity:		
Boar sperm motility inhibition	Paper I	Andersson *et al.*,1998
Bull sperm motility inhibition	Paper III	
Caco2 (colon carcinoma) cell exposure to cereulide	Paper III	
HeLa (cervical cancer) cell exposure to cereulide	Paper III	
Paju (human neuroblastoma) exposure to cereulide	Paper III	
Calu-3 (human lung carcinoma) exposure to cereulide	Paper III	
JC-1 staining for detecting electric transmembrane potentials in cells	Paper III	Reers *et al.*, 1995
Chemical methods for cereulide:		
LC-MS of cereulide	Paper II	Häggblom *et al.*, 2002
Methods for characterization of *B. cereus*		
Haemolysis	Paper I	
Bacillus cereus enterotoxin BCET-RPLA	Chapter 5.4	Beecher and Wong,1994
Anaerobic incubation in ≥ 99.5% N_2	Paper IV	
Anaerobic incubation in CO_2 9-13%, O_2 < 1%	Chapter 5.8.4	Oxoid (Cambridge, UK) anaerobic bags (Code: AN0035) Indicator code BR0055

5. Results and discussion

5.1 A new method for screening *B. cereus* isolates for cereulide production

In the present thesis I describe a novel rapid bioassay for detection of cereulide. The method is based on the inhibition of sperm motility within 5 min of exposure (Paper I). The test may be carried out with a single colony from the primary isolation plate with no need to prepare pure cultures for the toxicity assay. The toxicity threshold for the boar spermatozoa in this rapid assay was 2 ng of cereulide per ml. Boar semen for artificial insemination is commercially produced through out the year and therefore readily available.

Steps in the rapid boar sperm microassay

1. A loopful (about 10 mg wet wt) of biomass is picked from a single colony on an agar plate (e.g. 28 °C, 20-24 h) and suspended in 200 µl of methanol in a capped tube (about 4 ml)
2. The tube is capped and placed in boiling water for 15 min.
3. The tube is cooled and then vortexed for 2 min.
4. Aliquots of 0.5 - 10 µl of the obtained suspension are dispensed into 200 µl of extended boar semen and incubated in a thermoblock at 37 °C.
5. After 5 min of exposure, the motility of the sperm cells is recorded visually, using a phase-contrast microscope.

To date cereulide is the only heat-stable food poisoning toxin known to inhibit sperm motility within an exposure time of only 5 min. The other toxins of *B. cereus* inhibit sperm motility only when the exposure time is much longer. This fact explains why the outcome of the rapid boar sperm microassay is specific to cereulide.

We used the rapid sperm microassay to search for toxic *B. cereus* strains in a pasta food incriminated in a food poisoning case (Paper III). A toxin positive result (= inhibited sperm motility) was obtained for 83 strains of the 122 tested. Two researchers prepared the extracts and executed the microscopic analyses during one working day. In the earlier version of the boar sperm test (Andersson *et al*., 1998), the same task would have required much longer periods of time (Table 10).

Later, Rajkovic *et al.* (2006b) used a variant of our method, based on computer-aided boar semen motility analysis (Hamilton Thorne Ceros 12.1, Hamilton Thorne Biosciences, Beverly, MA, USA) for cereulide detection. In their protocol, the calibration curve was limited to the concentration range of 20-400 ng of cereulide per ml of the extracted bacteria or food. The boar sperm test is also used in Norway at the laboratory of P. E. Granum (Department of Food Safety and Infection Biology, Norwegian School of Veterinary Science, Oslo) which serves as the national reference laboratory for *B. cereus* (From *et al.,* 2007).

Table 10. The originally described sperm assay (Andersson *et al*., 1998) and the rapid sperm assay (Paper I): comparison of the essential features.

	Sperm assay (Andersson *et al*., 1998)	Rapid sperm microassay in this thesis
Culture time (20-30 °C)	10 days	1 day
Biomass needed (wet wt)	≥ 100 mg	5-10 mg
Time to make one extract	ca. 10 h	15-30 min
Exposure time	1-4 days	5-15 min
Detection limit	1 ng cereulide per test	0.4 ng cereulide per test

5.2 LC-MS-based quantative analysis of cereulide

The bioassays measure the toxic effects and not the toxic substances, but the quantification of toxin requires chemical methods. In my thesis, I executed a conclusive analysis of cereulide, using LC-MS (Papers I, II). HPLC was used for separation and detection was performed with ion trap MS. The column used was a Discovery C-8, 100 mm × 2.1 mm and 5-µm particle size (Sigma-Aldrich Corp., St. Louis, Mo, USA). The mobile phase consisted of 95% acetonitrile with 4.9% water with 0.1% trifluoroacetic acid at a flow rate of 0.15 ml min^{-1} at 25 °C. The effluent from the HPLC was fed into an electrospray ionization (ESI) ion trap mass analyser. My protocol for quantification differed from that described by Häggblom *et al*. (2002), in that I used specific ions rather than integration of the total chromatogram over a mass range of 500-1300 m/z. I used ion ranges (m/z) of 1153-1155, 1170-1172, 1175-1177 and 1191-1193. These are specific for the molecular adducts of cereulide with $[H^+]$, $[NH_4^+]$, $[Na^+]$ and $[K^+]$, respectively.

Using the above method, we analysed hundreds of specimens for their cereulide contents (examples shown in Table 11). The specimens represented a wide range of geographic and material origins. Table 11 shows that the toxicity titer measured with the rapid boar sperm

microassay corresponded very well with the actual cereulide concentration measured with LC-MS. As is seen from Table 11, the cereulide content of the bacterial biomass varied widely among the strains even when grown under identical conditions. Low producer strains contained only a few nanograms of cereulide per mg of *B. cereus* biomass (wet wt), while other strains produced up to 1000 times more cereulide. The method developed by me (this thesis) was subsequently used in other studies in our laboratory and elsewhere. The studies also showed wide differences in cereulide productivity among strains (Apetroaie *et al*., 2005; Carlin *et al*., 2006; Shaheen *et al.,* 2006). The reasons for the different productivities are as yet unknown, but they show that among the *B. cereus* emetic strains there are genetic and physiological differences that are important to recognize for the purpose of eliminating high cereulide producers from food.

Table 11. Cereulide production and affiliation of the *B. cereus* strains studied in this thesis. The strains were grown on trypticase soy agar plates.

B. cereus	Sperm microassay	Chemical assay	Origin of the strain, reference
Strains from food implicated or not implicated with illness	ng of cereulide	per mg of bacteria	
B116	190	150, 190, 230	Meat pastry, control sample (not food poisoning) Finland (Paper II)
B203	360	250, 360, 380	Rice mush, control sample (not food poisoning) Finland (Paper II)
B208	100	100, 120, 120	Cake, food poisoning, Finland (unpublished)
F4810/72	320	320, 380, 410	Food poisoning, UK (Turnbull *et al*., 1979)
B 347	300	320, 350, 350	Pasta dish, food poisoning, Finland (Paper III)
B308	1000	900, 1000, 1500	Risotto, food poisoning, Finland (Apetroaie *et al.*, 2005)
B412	500	420, 450, 500	Cake, food poisoning, Finland (Apetroaie *et al.*, 2005)
F5881/94	500	320, 400, 450	Fried rice, UK (Andersson *et al.,* 1998)
B117	< 0.9	< 0.2	Meat pastry, control sample (not food poisoning) Finland (Paper II)
F528/94	< 0.9	< 0.2	Beef chow mein and rice, UK (Pirttijärvi *et al.*, 1999)
Environmental isolates			
LKT I/1	400	350, 400, 500	Filling material of a building with moisture damage, Finland (Apetroaie *et al*., 2005)
7pk4	50	30, 50, 80	Indoor wall of a hospital with moisture damage, Finland (Apetroaie *et al*., 2005)
NS58	1500	900, 1000, 1100	Live Norway spruce, Finland (Hallaksela *et al.*, 1991)
NS88	1500	1000, 1500, 1700	Live Norway spruce, Finland (Hallaksela *et al*., 1991)
NS115	1000	700, 900, 1000	Live Norway spruce, Finland (Hallaksela *et al*., 1991)
NS117	1000	1100, 1200, 1200	Live Norway spruce, Finland (Hallaksela *et al.*, 1991)
Human and clinical isolates			
NC7401	300	380, 400, 400	Food poisoning patient, Japan (Agata *et al.,* 1994)
RIVM BC 00067	20	20, 25, 40	Human faeces, The Netherlands (Apetroaie *et al.*, 2005)
RIVM BC 00068	40	60, 80, 80	Human faeces, The Netherlands (Paper I)
RIVM BC 00075	100	100, 120, 150	Human faeces, The Netherlands (Apetroaie *et al*., 2005)
IH 41385	10	5, 5, 10	Dialysis fluid of dialysis patient, Finland (Andersson *et al.,* 1998; Ehling-Schulz *et al*., 2006)
B. cereus type strain			
ATCC 14579T	< 0.9	< 0.2	

5.3 Method for direct extraction and analysis of cereulide in foods

We designed a method for extracting and analysing the emetic toxin, cereulide, from food and applied this method in paper II to industrially manufactured bakery products (Table 12). The assay developed, based on solvent extraction, was optimized using a robotized extraction instrument. The best yield (> 70%) was obtained by extracting the bread with methanol-pentane (1:1) at a temperature of 100 °C and pressure of 103.4 bar (Paper II, Table 1).

For assessment of the toxicity of the food extracts, we used the rapid boar sperm microassay described in Paper I. It was possible to quantitatively measure cereulide in extracts of food, containing a myriad of substances, when cereulide-specific molecular ions were used (paper II) to minimize matrix interference. Internal calibration standards were spiked into the food matrix. The calibration curve was close to linear from 0.01 to 10 μg of valinomycin per ml in the bread extract. The detection limit was 2 ng of cereulide per g of bread. I used valinomycin as an internal and/or also as an external standard to assess the efficiency of extraction from the complex matrices such as food. The specific ions that I used to quantify the internal standard valinomycin were m/z 1111-1113, 1128-1130, 1133-1135 and 1149-1151.

Our developed method, specific for cereulide and based on LC-MS, has since been applied in various materials in many research projects at our laboratory: minced meat pasta food: (Pirhonen *et al*. 2005); infant food formulas (Shaheen *et al*., 2006) and paper pulp (Hoornstra *et al*., 2006). We also initiated collaboration with laboratories in several countries. Our method for foods was adopted by Carlin *et al*. (2006); Rajkovic *et al*. (2006b); Svensson *et al*. (2006); and Thorsen *et al*.(2006).

After our work was published in 2003, Hormazābal *et al.* (2004) described an LC-MS-based quantification of cereulide in two foods: figs and rice. Their method of extraction was more elaborate than ours. They reported as the detection limit 1 ng of cereulide per g of food and as the quantification limit 2 ng of cereulide per g of food. This is similar to the results of our method: 2 ng of cereulide per g of food. Their extraction method was based on a mixture of acetone-tetrahydrofuran, methanol and water. The organic layer was separated from the aqueous layer by adding chloroform. The method thus required solvents highly toxic to humans (tetrahydrofuran, chloroform), whereas we used less hazardous solvents. Hormazābal

et al. (2004) used only one specific ion, the NH_4^+ adduct, m/z 1170.9, for cereulide detection. We used four different cereulide-specific ions (chapter 5.2), corresponding to adducts of K^+, Na^+, H^+ and NH_4^+. In our opinion this is needed for accurate assays, because the adduct ratios may vary between different food matrices and analyses.

Recently, we began to use ethanol as the solvent for cereulide extraction. Ethanol is less toxic to humans than methanol and less explosive than pentane. Ethanol turned out to be at least equally as effective as the previously used solvents. In this method, the plain food (usually 1-10 g) is flooded with ethanol and allowed to equilibrate in a stationary position in a closed jar at room temperature (21-23 °C) overnight. The ethanol phase is then harvested and is evaporated to dryness at 50 °C. After all liquid evaporated, the residue is dissolved in 1 ml of ethanol or methanol.

5.4 Mining for cereulide producers from food

We examined various bakery products (not implicated with illness): meat pastry, rice pastry, white bread and whole-grain wheat bread for the presence of toxin-producing strains of *B. cereus* (Table 12). Of each food item, five parallel products were acquired from manufacturers or from the consumer markets. Before analysis, the foods were preheated (72 °C, 5 min) to activate the spores and then stored for 4 d at room temperature (21-23 °C). Before and after storage, parallel aliquots were combined, mixed and streaked on bovine blood agar and cultivated for 1 d at 28 °C. Colonies with a morphological appearance resembling that of *B. cereus* (i.e. *sensu lato*), were selected for the study. From the two types of pastry, 20 colonies with *B. cereus*-type morphology were selected for toxicity analysis using the rapid boar sperm microassay, the LC-MS method (cereulide) and by commercial kit for haemolysin BL. From the meat pastries 14 of the 20 tested (70%) weakly haemolytic isolates were toxic in the sperm assay and one (5%) of the 20 from the rice pastries. LC-MS analysis confirmed that the toxic compound was indeed cereulide. The diarrhoeal HBL enterotoxin was produced by other *B. cereus* strains from the same rice and meat pastries. These results show that industrially prepared pastries may contain cereulide producers. In paper II we showed that the rice and meat pastries supported cereulide production when the producer strains were present. In contrast to meat and rice pastries we found no toxic *B.*

cereus from any of the breads (Table 12). Pirhonen *et al*. (2005) also described a food that contained both diarrhoeal (HBL) and emetic toxin (cereulide) producers.

The plate-culturing medium first used for determining the pathogens from foods incriminated with food poisoning incidents is often blood agar incubated at 30 °C (Parry *et al.,* 1983) or at 35 °C (FDA, 1998). The colonies used for pathogenicity testing are usually picked from overnight-grown plates based on the characteristic *B. cereus* colony morphology, size and zone of clear haemolysis (reviewed in the doctoral thesis of Pirttijärvi, 2000). We determined that emetic toxin-producing isolates were found exclusively among colonies with low (clearing zone of ≤ 2 mm) or no haemolytic activity (i.e. no clearing zone) on plates with 5 vol % of defibrinated bovine blood (Paper I, Figure 1). We found not a single isolate with a wide, clear zone of haemolysin that would produce cereulide, although > 200 isolates were tested (Paper I). This demonstrated that only by choosing the strongly haemolytic colonies from the primary plate culture are the cereulide-producing strains likely to be excluded.

Table 12. Mining for spore-forming toxic bacteria and *B. cereus* from industrially manufactured bakery products for day of purchase and after storage of 4 d at 21-23 °C. Most spore-forming bacteria belong to the *B. cereus* s.l.group (based on type of colony morphology). Cereulide production was measured with the boar sperm microassay and the presence of cereulide with the LC-MS analysis. Enterotoxin haemolysin BL production was detected with an immunoassay (Oxoid kit).

	Total colony count of spore-forming bacteria (cfu/g)		**Colony count of *B. cereus s.l.* (cfu/g)**		**Isolated emetic strains*/ all tested *B. cereus s.l.* strains**	**Isolated diarrhoeal strains** / all tested *B. cereus s.l.* strains**
Bakery products	on day 0	on day 4 after storage	on day 0	on day 4 after storage	4 d	4 d
White bread	10-100	10^3	10	100	0/ 7	0/ 5
Whole grain-wheat bread	10-100	10^3	0	0	0 /0	0/ 0
Meat pastry	10^3	10^8	100	10^8	14/ 20	1/ 20
Rice pastry	10^3	10^8	100	10^8	1/ 20	2/ 20

*) positive for both toxicity (sperm test) and the production of cereulide

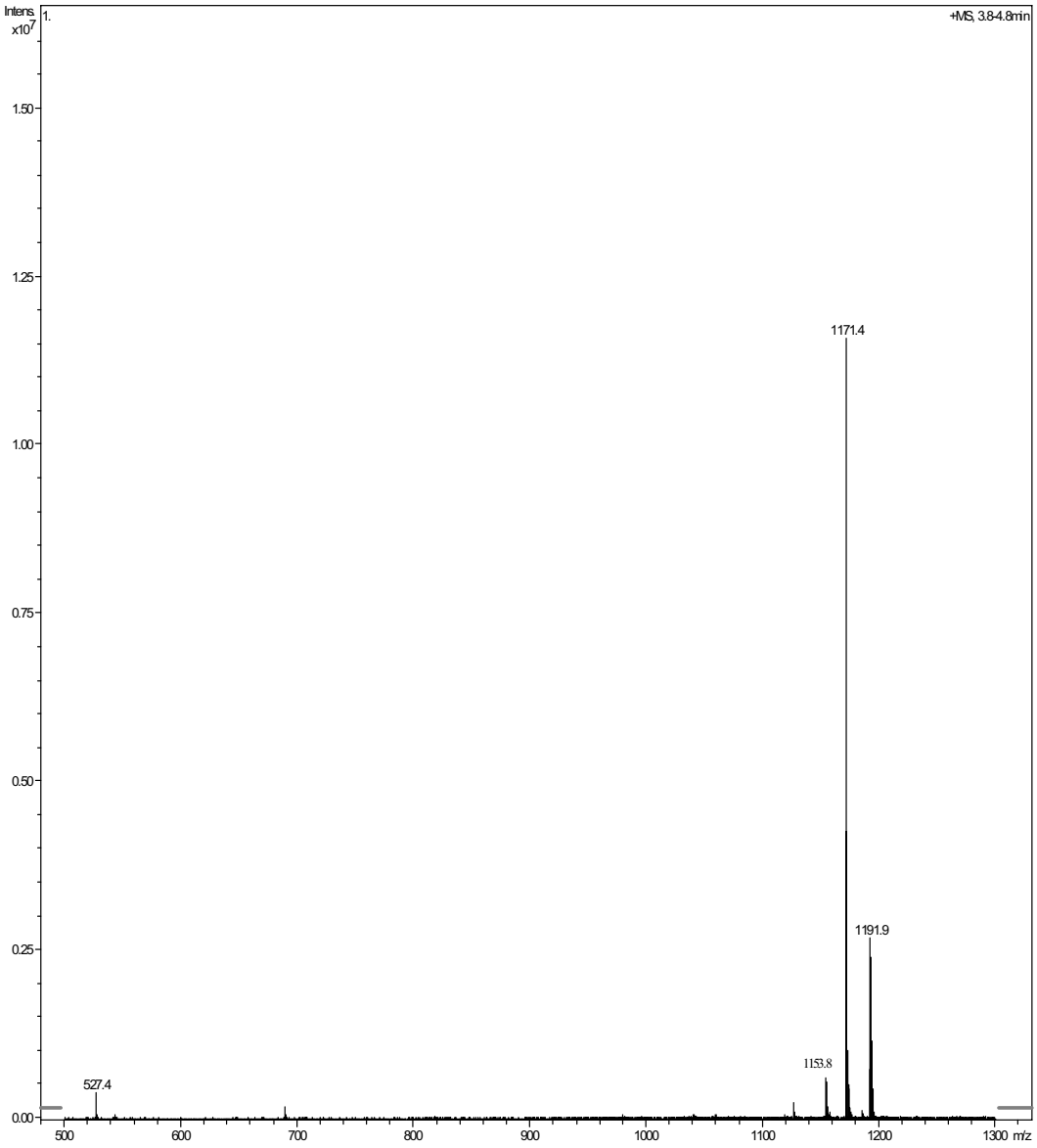
**) positive in the HBL test

5.5 Cases of emetic *B. cereus* food poisoning

Using the method newly developed in this thesis for analysing cereulide directly from foods (Paper II), we analysed the remains of a pasta dish consumed by two adult persons subsequently taken sick by emetic illness. Local authorities were able to rescue the remains of the poisonous meal and first plated the suspected food as usual (chapter 5.4) and selected a few *B. cereus* resembling colonies for preparing pure cultures. These strains all produced diarrhoeal toxins; none produced cereulide. However, the illness symptoms of the affected persons indicated the emetic syndrome and our laboratory was therefore called in to restudy this food in collaboration with the Finnish National Veterinary and Food Research Institute EELA (since 2006 renamed the Finnish Food Safety Authority, EVIRA). A total of 122 *B. cereus* isolates were randomly sampled from this food and over half of these (68%) produced the emetic toxin, as shown by the rapid boar sperm microassay (Paper I; Pirhonen *et al*., 2005). The remains of the consumed meal were then solvent-extracted directly by both the manual (Andersson *et al*., 1998) and the robotized (Paper II) protocol. The same type of meal, not associated with the food poisoning, was purchased from a local store for reference. The toxicity titers of both dishes were determined by the boar sperm microassay.

The results showed that the manually prepared extract of the illness-incriminated food contained 1 - 2 μg of cereulide equivalents of the emetic toxin per g of the suspected food. The extract prepared with the robotized method, contained 1.5-3 μg of cereulide equivalents of the emetic toxin per g of the food. When the substance, cereulide, was quantitated by the calibrated LC-MS method, 1.4 μg g^{-1} were found in the manually prepared extract and 1.7 μg g^{-1} in the robotized extract. Mass spectrum of robotized extracts is shown in Figure 3. The reference food contained cereulide below the detection limit of the LC-MS method, which for that food was 0.01 μg cereulide g^{-1}. The illness-affected persons thus had consumed ~170 μg of cereulide per each 100 g of the ingested food. The illness-causing dose thus may have been ≤ 8 μg kg^{-1}(60 kg), more likely 2 - 5 μg kg^{-1}, if the amount of the food actually ingested did not exceed 100 - 200 g per person. Our results thus show that humans are very sensitive to cereulide, as are the rhesus monkey and musk shrew (Review of the literature, Table 6) and much (50-100 ×) more sensitive than mice.

Figure 3. Mass spectrum of cereulide in the poisonous meal. The main adduct of the molecular ion was NH_4^+, with m/z values 1171.4, followed by $M+K^+$ (1191.9) and $M+H^+$ (1153.8). The Na^+ adduct was the smallest and is not visible in the figure.



5.6 The case of acute liver failure

We analysed the cereulide contents in a human liver sent to us from Belgium. The liver had been removed from an infant patient in a Belgian hospital. At the time of arrival, the liver had already been preserved in formaldehyde to prevent decay during transport. The infant, 11 months of age, suffered from liver failure (steatotic liver) with a suspected association with food poisoning. The child had been in hospital care for many days before removal of the liver. The physician suspected possible food poisoning by emetic toxin-producing *B. cereus*, but no food was available for analysis.

We used porcine liver (purchased from the local store) as a model to design a method for the extraction and analysis of cereulide from a formalinized liver. The method we developed was as follows:

1. Washing

   2 g of the fresh liver were soaked in 20 ml of distilled water at room temperature overnight. The water was changed and the formalinized liver tissue stored another night at room temperature.
2. The water was drained and the liver tissue dried at 60 °C.
3. The liver tissue was ground under liquid nitrogen in a mortar.
4. The dry liver powder was flooded with 10 ml of ethanol and incubated for 2 d at room temperature.
5. The ethanol phase was harvested and evaporated to dryness at 50 °C. When all liquid had evaporated, the residue was dissolved in 1 ml of methanol.
6. The methanol extract was stored at -20 °C until LC-MS analysis. The limit of detection after this extraction protocol by LC-MS was 5 ng of cereulide per g fresh wt of liver (obtained by calibration with cereulide-spiked formalinized porcine liver).

We analysed the child´s liver using this method, but the concentration of cereulide, if present, remained below the detection limit of 5 ng g^{-1}. Afterwards we realized that the lipophilic toxin, cereulide (log K_{ow} = 6), could already have migrated from the liver through the blood stream and into the body fat, which should have been available for analysis.

5.7 Analysis of the toxicity target of cereulide in mammalian somatic and germ cells

In our research group, boar spermatozoa have been used for detecting cereulide toxicity since 1998. The question arises whether the high toxicity of cereulide to boar spermatozoa is dependent on the animal species or whether it is specific for haploid, gametic cells like sperms. To answer this question we investigated the cereulide sensitivities of bovine and porcine sperm cells (Paper III). Both sperms were obtained as commercial products, purchased from the suppliers of sperm for farm use. The responses to cereulide of these sperm cells were compared with those of the commercially available human somatic cell lines cervical cancer (HeLa), colon carcinoma (Caco-2), lung carcinoma (Calu-3) and a research cell line, neural cell (Paju). HeLa is one of the most widely used cell lines in the world. The Caco-2 cells were used to model the contact of cereulide in food with the human digestive epithelia tract and Paju cells to assess the potential for neurotoxicity. The Calu-3 cells were used to model exposure to inhaled toxin. This was done because cereulide-producing bacteria are known to occur in moisture-damaged buildings where the occupants suffer from building-related illness (Andersson *et al.,* 2002).

The effects of cereulide on the membrane potentials of the mitochondria ($\Delta\psi_m$) were visualized by staining with the membrane potential-sensitive dye JC-1. The results showed that the threshold concentration of cereulide for dissipating the $\Delta\psi_m$ was similar in the four types of cultured human somatic cells and in the boar sperm cells: 2 ng of cereulide per ml. The sperm cells in bull semen tolerated over 100 times more cereulide than did those in the boar semen. Commercially available bull semen is sold in frozen form and stored in liquid N. As such it contains an extender with cryoprotectants. In contrast, boar semen is sold unfrozen and its extender contains no cryoprotectant. The toxicity of cereulide may have been attenuated by the freeze-preserving additives rich in protein and lipid. Lipids are known to attenuate the toxicity of lipophilic bioactive substances as explained by Seibert *et al*. (2002). Cereulide is highly lipophilic with a log $K_{ow} = 6$ (Teplova *et al.,* 2006). The cell density, exposure conditions, cultivation method (suspension or monolayer) affect the sensitivity of the exposed cells to toxins. It is also known that malignant cell lines may be less sensitive than primary cells (Paananen *et al.,* 2002; Teplova *et al.*, 2004 and Andersson *et al*., 2007).

My results indicate that cereulide is a rapidly acting (minutes to hours) universal poison, to which all mammalian cells are sensitive, germ cells as well as somatic cells.

5.8 Cereulide production under different environmental conditions

This chapter deals with factors affecting cereulide accumulation in artificial media or in foods and seeding with emetic strains of *B. cereus.*

5.8.1 Cereulide production by emetic *B. cereus* in laboratory cultivation media (cereulide contents of the harvested bacterial biomass)

My research showed that the content of cereulide in *B. cereus* biomass was strongly modulated by the growth environment of the bacterium; Table 13 summarizes the results. The cereulide content of *B. cereus* biomass harvested from rich agar media (tryptic soy agar, brain heart infusion agar and blood agar) was high, 220-450 μg g^{-1} of biomass wet wt. Lower amounts of cereulide (22 - 71 μg g^{-1} wet wt) accumulated when the same emetic *B. cereus* strains were grown on medium-rich agar (MYP, R2 agar or rice-water agar). *B. cereus* grew well on MYP agar (composed of mannitol, egg yolk and polymyxin B agar), equal to levels found in the richest media. On R2 agar (composed of yeast extract, peptone, casamino acids, dextrose, starch, sodium pyruvate, dipotassium phosphate and magnesium sulphate), as well as on rice-water agar, *B. cereus* formed less dense, but clearly visible, colonies. Adding L-leucine and L-valine (0.3 g l^{-1}) stimulated cereulide production 10 - 20 - fold on R2 and rice-water agar (Paper IV). This increase in cereulide production was induced by the free amino acids (L-leucine and L-valine) but not peptide-bonded amino acids. This was documented by adding peptone containing similar amounts of peptide-bonded L-leucine and L-valine: there was no effect on cereulide production. These amino acids, L-leucine and L-valine, are also approved food supplements (flavour modifiers) in the USA [http://jecfa.ilsi.org /evaluation.cfm (4.10.2007)] and in the EU (European Commission, 2006). The Scientific Panel has not considered food supplement effects for microbial toxin production (European Commission, 2006).

5.8.2 Time course of cereulide production

The question often asked is when cereulide production begins and finishes in different media. Figure 4 shows the time course of cereulide accumulation by independent isolates of emetic *B. cereus* from live Norway spruce (*Picea abies*). The strains were isolated with aseptic equipment from live trees in the forest during the coldest period in winter (Hallaksela *et al.,* 1991). *B. cereus* strains NS 85, NS 88, NS115 and NS 117 were grown on tryptic soy agar 7 d at room temperature (21-23 °C) and the biomass obtained analysed for cereulide. In two of the strains, the concentrations of cereulide in the biomass continued to increase for 3 d and in two other strains for 6 d, indicating that individual strains, although of the same origin, may have different kinetics of cereulide production. Decrease in the cereulide content of the *B. cereus* strains when the cultures became 6 - 7 d old indicates that cereulide may be autodegraded by its producer strains.

Table 13 also shows the results for liquid laboratory media and milk. Cereulide production by *B. cereus* strains B116, B203 and F4810/72 in trypticase soy broth mainly started 16 h after inoculation at 22 °C. After 65 h the concentration of cereulide in the broth rose to 3-6 µg ml^{-1}. Sporulation of *B. cereus* on tryptic soy agar begins after ~ 48 h. These results are in line with those of Häggblom *et al.* (2002), who showed that cereulide accumulation in broth cultures started as soon as the culture reached the stationary phase, i.e. before the culture sporulated, and then remained at a plateau concentration for the subsequent 24 h.

Based on our results the final concentration of cereulide was probably reached overnight on rich solid media, such as brain heart infusion agar, whereas in tryptic soy broth cereulide production only started after 16 h of incubation at 22 °C. The results further indicate that the onset of cereulide production occurred sooner on solid culture media than in liquid medium - explaining why cereulide food poisonings apparently have never been reported for liquid foods.

5.8.3 Cereulide production in foods

I investigated foods to determine their amenability to accumulate cereulide (Table 13). I found that rice pastry and meat pastry (seeded with *B. cereus* strains F4810/72, B116 or B203) accumulated 0.7-5.5 µg of cereulide per g within 4 d (Table 13). The pastries contained rice and proteinaceous additives. Plain boiled rice also accumulated large amounts, 2 - 4 µg per g of food, of cereulide. Rice alone aparently contains sufficient amounts of the essential amino acids (threonine, leucine and valine) to maintain growth of *B. cereus* (naturally auxotrophic for these amino acids) and cereulide production. I found that the cereulide-producing strains studied (B116, B203 and F4810) survived the heating applied during baking of pastries ≥ 20 min at 250 ºC (dry heat) and cooking of food (boiling for ~ 30 min). Wijnands *et al.* (2006) found that rice- and pasta-containing dishes (ready-to-eat foods) mostly contained ≥ 10^5 cfu of *B. cereus* per g sampled under normal retail conditions. The dose of *B. cereus* inoculated in the foods in our studies, 10^6 cfu g^{-1}, was therefore realistic.

I cooperated with Andreja Rajkovic by analysing the food samples from his study for cereulide by the LC-MS method. The samples were pasta, potato puree, milk and rice inoculated with *B. cereus* strains NS117 and 5964a (Rajkovic *et al*. 2006b). I found 2 µg g-^{1}of cereulide in the rice, which is similar to what I found earlier in rice (Paper IV). I found high amounts of cereulide in the potato puree and pasta (after 48h shelving time at 28 °C) sent to me by A. Rajkovic, 4 and 3 µg g^{-1}, respectively, clearly showing, that these foods are sensitive to cereulide production.

Cereulide production in milk is an interesting topic. I found that no cereulide (< 0.5 ng ml^{-1}) was produced in shaken consumer skim milk at 22 °C (Table 13), even though it had been seeded with 10^6 cfu of cereulide producer strains 4 d earlier. My studies with Andreja Rajkovic (2006b) showed that no cereulide accumulated in shaken milk at 28 °C after 48 h. However, I found in the same study that the same strains of *B. cereus,* NS117 and 5964a, produced 1 µg ml^{-1} in whole consumer milk that had been shelved stationary.

Agata *et al.* (2002) reported different results from Japan: shaken milk was more toxic in the HEp-2 cell assay (0.64 µg cereulide equivalents per g) than stationary (< 0.01 µg g^{-1}) incubated milk. In the protocol of Agata *et al.* (2002), the *B. cereus* was grown in milk for

24 h, centrifuged, the supernatant collected and autoclaved and the toxicity titer measured in the supernatant. Their results (Table 4, Agata *et al.,* 2002) show that after 24 h the *B. cereus* strains in the stationary incubated milk culture was still growing, whereas the shaken culture had already attained the maximal cfu content. In my study *B. cereus* was grown for 4 d (Table 13) or 48 h (study done with A. Rajkovic, 2006b). At these times, the static cultures should also have been fully grown. A further point needing emphasis, is that cereulide is insoluble in water (log K_{ow} = 6, Table 6). Consequently, the toxin produced will likely remain bound to the bacterial cells or their debris, or (in the case of milk) accumulate in the fat and float in the supernatant. Therefore the outcome for skim milk will be different from that of whole milk. In my study, I applied no centrifugation step and the whole-milk sample was extracted with an organic solvent suitable for solubilizing cereulide.

The first report on *B. cereus* emetic toxin in milk (or liquid growth media) is that of Szabo *et al.* (1991). Later, other researchers (Sakurai *et al.*, 1994; Agata *et al.* 1996,1999, 2002; Finlay *et al*., 1999) followed the Szabo protocol. In this protocol the liquid culture was centrifuged, the pellet discarded and the supernatant boiled or autoclaved (to destroy the heat-labile toxins and viable bacteria) before the toxicity assay. In such a protocol, the toxin bound to the bacterial cell pellet, or to the food debris, is lost from the toxicity result. This is especially true for skim milk, where there is no fat to retain the toxin in the supernatant. In some protocols (Finlay *et al*., 1999), the centrifugation was done for 40 min at 4 °C, likely immobilizing most of the cereulide in the hydrophobic phase and on the walls of the centrifuge tubes. The protocol of Mikami *et al.* (1994) was different: the autoclaving step preceded the centrifugation. Mikami *et al.,* call their protocol an "improved method", leading to higher yields of cereulide, possibly because autoclaving lysed the cells, reducing the size of the cell pellet and thus increased the toxin yield in the supernatant.

Our study is the first in which the substance cereulide as well as its toxic effects were measured directly in food. All other studies published to date outside our laboratory used indirect methods, including bioassays on toxicity of heated *B. cereus* or of food extracts. Since no heat-stable toxin other than cereulide has so far been found in *B. cereus* from foods, all the studies published by Agata *et al.* (1999, 2002) and Szabo *et al.* (1991) most likely measured toxicity caused by cereulide.

Hormazābal *et al.* (2004) published an application of our earlier described LC-MS method, in which they determined the cereulide contents in figs and rice. These authors did not measure the toxicity from these samples. We believe that it is important to combine the LC-MS method with the bioassay method to be sure of the source of the toxicity. This was done for determination of cereulide from infant food formulas (Shaheen *et al.,* 2006).

5.8.4 Cereulide production under different atmospheres

Table 13 also summarizes the results in which cereulide productions in the same foods or media by the same strains of *B. cereus* were measured under different atmospheres. These results show that in > 99.5% N_2, no cereulide was produced in liquid laboratory media or in the two solid foods studied (rice, beans). However, the cereulide contents found after aerobic or anaerobic incubations of *B. cereus* strains on tryptic soy agar plates were also highly independent of the atmosphere: anaerobic (CO_2 9-13%, O_2 < 1%, in N_2) or ambient air.

Finlay *et al.* (2002) reported that the density of *B. cereus* strains F4810/72, F3748/75, F3744/75, F4562/75, F4552/75 and F2427/76, measured as viable counts after 24 h at 30 °C in skim milk medium, remained lower ($P < 0.01$) under anaerobic conditions than under aerobic conditions. Toxicity, presumably due to cereulide, was nondetectable in the anaerobic cultures, even though the viable counts were consistently > 10^6 cfu ml^{-1} which should have been sufficient for producing measurable amounts of cereulide (Finlay *et al.,* 2002).

Rajkovic *et al.* (2006a) found that when the atmosphere contained less than 1.6 vol % O_2 in N_2, no cereulide was produced by *B. cereus* growing on solid medium (tryptic soy agar 24 h, 28 °C), but ample cereulide was produced, about 1 µg g^{-1} biomass wet wt, on the same plates in an atmosphere of > 4.5% O_2 in N_2. The *B. cereus* strains used in that study were NS117 (Finnish spruce tree isolate from our laboratory) and 5964a (food isolate from a fatal case of *B. cereus* poisoning in Belgium).

Based on our results and those published by other researchers, the role of O_2 in cereulide production by *B. cereus* is not a simple one. An N_2 atmosphere in the absence of CO_2 did not allow cereulide production in the absence of O_2 but did in the presence of CO_2. However, the CO_2 or lowering of the redox -potential of the growth environment could have promoted

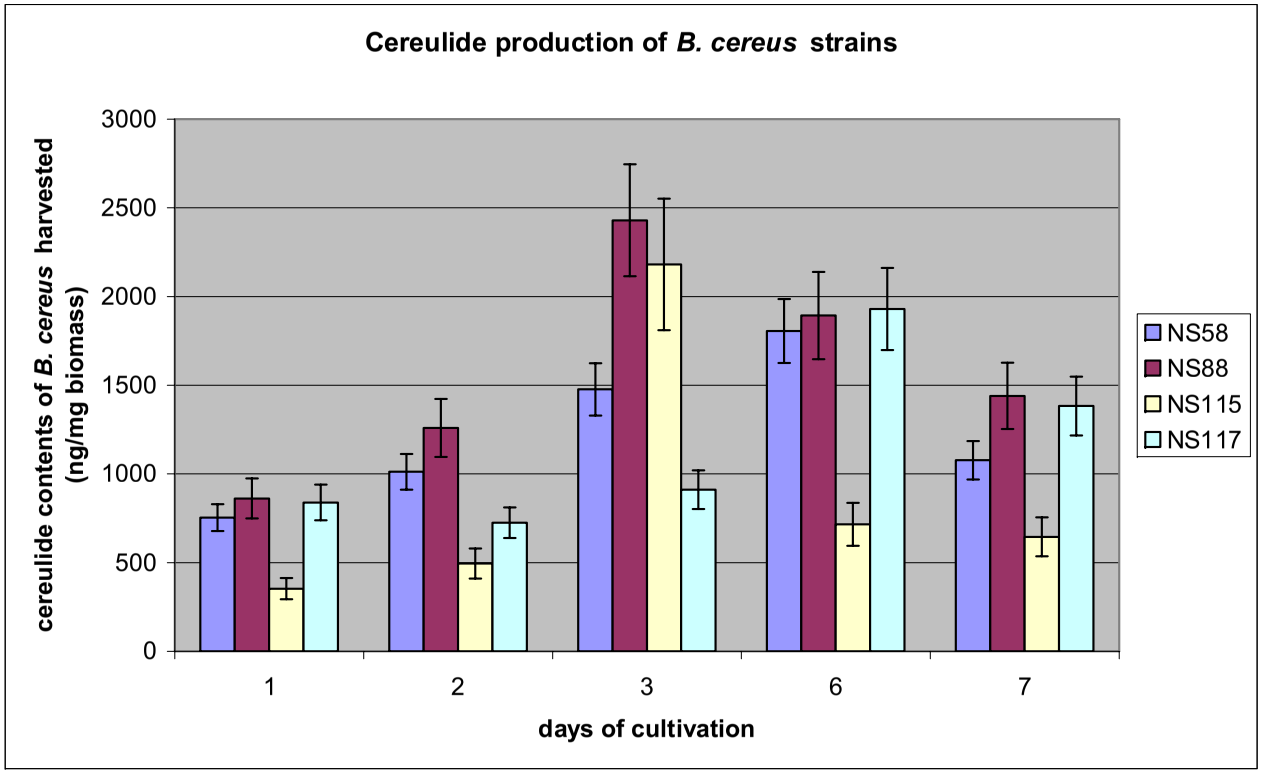
toxin production in facultative anaerobic *B. cereus*. Further studies are necessary along this line.

Table 13. Accumulation of cereulide in *B. cereus* biomass when grown in different cultivation media and in foods that were seeded with emetic *B. cereus* strains. B116 was isolated from meat pastry (Finland, a control sample), B203 from rice porridge (Finland, a control sample) F4810/72 from a food poisoning case (UK). All results are given as means of two or three independent replicate cultures of the same strain.

	Incubation at 22 °C		*B. cereus* strain		
Growth substrate	**days of growth**	**atmosphere**	**B116**	**B203**	**F4810/72**
Biomass of *B. cereus* harvested from			**Cereulide measured in the bacterial biomass (µg/g)**		
Tryptic soy agar	4	ambient	280	450	350
Tryptic soy agar	4	CO_2 9-13%, O_2 < 1% in N_2	310	400	320
Brain heart infusion agar	4	ambient	235	360	450
Blood agar	4	ambient	220	280	300
R2 agar	4	ambient	40	74	71
Mannitol egg yolk polymyxin B agar	4	ambient	25	50	22
Rice-water agar	4	ambient	ND	ND	28
Liquid media (shaking 120 rpm)			**Cereulide measured in the liquid (µg/ml)**		
Consumer skim milk (0% fat)	4	ambient	0.02	0	0.05
Consumer skim milk (0% fat)	4	> 99.5 vol % N_2	0	0	0.08
Trypticase soy broth	4	ambient	5	3.5	5.5
Trypticase soy broth	4	> 99.5 vol % N_2	0.01	0	0
***B. cereus*- inoculated foods**			**Cereulide in the food (µg/g)**		
White bread	4	ambient	0.02	0.03	0.03
Whole-grain wheat bread	8	ambient	0.01	0.02	0
Rye bread	21	ambient	0	0.01	0.01
Meat pastry, dough	4	ambient	0.8	0.6	0.3
Meat pastry, filling	4	ambient	0.7	4.2	5.5
Rice pastry	4	ambient	1.8	1.5	1.55
Beans, boiled	4	ambient	ND	1.6	0.7
Bean, boiled	4	> 99.5 vol % N_2	ND	0.1	0.06
Rice , boiled	4	ambient	3	2	4
Rice, boiled	4	> 99.5 vol % N_2	0.01	0.02	0.01

ND= not determined

Figure 4. Production of cereulide by *B. cereus* strains (isolated from Norway spruce) NS58, NS88, NS115 and NS117. The strains were grown aerobically on tryptic soy agar at 22 °C. The cereulide contents of independent replicates (three) were measured with LC-MS.



6. Conclusions

When I initiated my doctoral research in 2001, most published studies on *B. cereus* were focused on the production of various diarrhoeal enterotoxins. Here I focused on production of the *B. cereus* emetic toxin, cereulide, a known mitochondriotoxin. Significant outcomes of my work include the following:

1. We developed a rapid *in vitro* method to screen for the presence of the heat- stable *B. cereus* toxin for a large numbers of strains in a short time. This method is based on the rapid (5 min) effect of cereulide on boar sperm cells. We found that the toxin-positive *B. cereus* strains always had a phenotype of poor haemolysis on blood agar. This revelation was used to preselect the poorly haemolytic colonies for toxin analysis. This is contrary to the current practice in most laboratories, where the haemolytic *B. cereus* colonies are preferred.

2. We studied the toxicity threshold of cereulide for the human HeLa, Caco-2, Paju and Calu-3 cell lines. We found that the toxicity endpoint of cereulide for boar sperm cells and human cells was similarly low, showing a detection limit of 2 ng of cereulide per ml of cells. This indicates that the boar sperm assay is suitable for *in vitro* assessment of possible effects on human cells by extracts suspected of containing the mitochondrial toxin, cereulide, of *B. cereus*.

3. We designed a method for quantitative extraction of the *B. cereus* emetic toxin not only from the biomass of laboratory-grown *B. cereus*, but also directly from foods. Cereulide is a highly lipophilic substance and is practically insoluble in water. The novel extraction protocol is based on organic solvents. The extraction was optimized (100 °C, 103.4 bar) to effectively solubilize cereulide from bacterial biomass and from food.

4. The extracted cereulide was separated from other constituents by LC and then quantified based on the m/z values of cereulide-specific NH_4^+, H^+, Na^+ and K^+adducts. The bioassay for toxicity was performed on the same extract, using the boar sperm microassay. This double protocol verified that cereulide is the toxin identified and that it preserved its biological activity (toxicity) despite the aggressive extraction method.

5. Using the new method for cereulide quantification, we were able to disclose the dose of cereulide causing illness for healthy adult persons. We analysed the actual remains of the meal implicated in an outbreak of cereulide poisoning, using the new boar sperm microassay and the novel chemical assay based on LC-MS. Both methods showed that pasta contained 1.5 - 1.7 μg of cereulide per g. Ingestion of 100 g of such food means exposure to 150-170 μg of cereulide. My report was the first worldwide in which the dose causing a serious acute vomiting syndrome in humans was established. My results showed that the acute illness-causing dose is lower for cereulide than that for any other known microbial heat-stable toxin.

6. I studied several industrially manufactured foods to determine their susceptibility to accumulated cereulide. I found that rice, rice-containing pastries and beans accumulated high concentrations of cereulide, 0.3 - 5.5 μg g^{-1}, when stored at nonrefrigerated temperatures for up to 4 d. My results show that if emetic *B. cereus* strains are present in food, the risk of food poisoning cannot be overlooked when nonrefrigerated products, such as bakery products, are eaten days after manufacture.

7. A direct cereulide-specific assay made it possible to identify environmental factors promoting or preventing the production of this toxin. I found that *B. cereus* emetic strain (F4810/72) produced 450 μg of cereulide per g of cells (wet wt) on brain heart infusion agar during 4 d at room temperature. Under similar conditions, *B. cereus* (F4810/72) produced only 22 μg of cereulide per g of cells (wet wt) on mannitol egg yolk agar, 28 μg on rice-water agar and 71 μg on R2-agar. Adding the free amino acids L-leucine and L-valine stimulated cereulide production on oligotrophic R2 agar and/or rice-water agar 10 - 20 fold. Interestingly, adding meat peptone (5 g l^{-1}), containing the same amount of (peptide-bonded) amino acids (0.3 g L^{-1}) in the same medium promoted growth of the toxin producer, but had no significant effect on cereulide production. L- valine and L-leucine are approved food supplements and widely used as free amino acids in food technology.

8. Storage of *B. cereus* cultures or foods under N_2 atmosphere (> 99.5 vol % of N_2) prevented the production of cereulide for 4 d. But when CO_2 was present, the absence of O_2 did not prevent the production of cereulide. This may indicate that CO_2 or lowering of the redox potential promoted toxin production, but further studies are needed.

9. The actual production of cereulide was strongly strain-dependent; 5-1700 ng of cereulide per mg of *B. cereus* biomass (wet wt). Therefore, it is not possible to predict the toxic potential of any foods based only on the presence and density of the cereulide synthase gene as measured by quantitative PCR.

10. Emetic toxin-producing *B. cereus* strains can readily be detected in rice-containg pastries several days after baking. I found that several cereulide-producing strains (B116, B203 and F4810) survived the heating applied during baking of pastries ≥ 20 min at 250 ºC and cooking of food (boiling for ~ 30 min).

7. Tiivistelmä

Suomessa rekisteröityjen ruokamyrkytysepidemioiden määrä on vaihdellut samoissa luvuissa rekistereiden koko pitoajan, 40-90 epidemiaa ja 1000-9000 ruoasta tai juomavedestä sairastunutta henkilöä vuosittain. Näin siitä huolimatta että hygienian keinot, mm. kylmäketju on tuona aikana parantunut. Vuoteen 2004 saakka salmonella ja sitten kampylobakteeri olivat bakteeriepidemioiden pääasialliset aiheuttajat, mutta viime vuosina 2005–2006 *Bacillus cereus* nousi yleisimmäksi. Samantapainen kehitys alkoi mm. Saksassa jo 1990 luvulla. Yksi syy tähän kehitykseen saattaa olla *Bacillus cereuksen* tuottaman oksetustautia aiheuttava toksiini, kereulidi. *Bacillus cereus* on luonnossa ja elintarvikkeiden raaka-aineissa hyvin yleinen bakteeri. Toisin kuin salmonellat ja kampylobakteerit, se tuottaa itiöitä jotka kestävät pastöroinnin ja keittämisen sekä toksiinia joka kestää jopa höyryautoklavoinnin. *Bacillus cereus* itiöt aiheuttavat ruokamyrkytysriskin kuumennetuissa elintarvikkeissa ja ruoissa joita ei syödä valmistuspäivänä, koska ruoan jäähtyessä itiöt muuttuvat kasvullisiksi bakteerisoluiksi ja voivat tuottaa toksiineja. Tämän väitöskirjatyön aihe oli kereulidi ja sitä tuottavien *Bacillus cereus* kantojen tunnistaminen, mittaaminen ja kereulidin tuottoon vaikuttavat tekijät.

Kehitin menetelmiä kereulidin mittaamiseksi suoraan elintarvikkeesta. Määrittämisen edellytys oli toksiinin kemiallisten ja fysikaalisten ominaisuuksien tuntemus, jotta saatoin suunnitella menetelmän toksiinin tehokkaaseen eristämiseen elintarvikkeesta ja raaka-aineesta. Koska kereulidi ei liukene lainkaan veteen, käytin uuttokemikaalina orgaanisia liuottimia, metanolia, etanolia ja pentaania. Leipomo- ja konditoriatuotteista uutin kereulidin korkeassa lämpötilassa (100°C) ja paineessa (103.4 Bar). Vaihtoehtoisesti uutto voidaan suorittaa kuivattamalla elintarvike ja uuttamalla sitä elintarvikkeen tilavuuteen nähden kaksinkertaisessa pitoisuudessa etanolia noin 12 tuntia. Tätä menetelmää käytin mm. pastalle ja perunasoseelle. Nestemäiset elintarvikkeet, kuten maito, voidaan uuttaa pentaaniin tai kuivattaa ja suorittaa etanoliuutto. Nämä uuttomenetelmät ovat tärkeä parannus kereulidin aiheuttaman ruokamyrkytysriskin tutkimukselle, sillä ennen kereulidi uutettiin niin tuottajabakteerista kuin elintarvikkeestakin veteen jolloin kereulidi saanto oli huono ja vaihteleva riippuen elintarvikkeen rasvaisuudesta.

Kun mikrobin aiheuttamaksi epäiltyä ruokamyrkytystä selvitetään, pitää osata todeta kaksi asiaa. Ensimmäiseksi epäillyn elintarvikkeen todellinen myrkyllisyys. Monet mikrobimyrkyt, vaikkakaan ei kereulidi, inaktivoituvat elintarvikkeen käsittelyprosessin aikana esimerkiksi kuumentaessa tai hapottamalla etikalla. Toiseksi myrkyn kemiallinen tunnistaminen. Siis onko kyseessä kereulidi vai jokin muu lämpökestoinen aine, esim. homemyrkky eli mykotoksiini. Tämä tieto tarvitaan myrkyn alkuperän tehokkaaseen selvittämiseen. Myrkyllisyyden toteamiseen kehitin työtoverini Maria Anderssonin kanssa pikamenetelmän, jonka avulla kereulidi voidaan todeta 5-15 minuutissa. Kehittämällämme testillä voidaan nopeasti todeta mikä mahdollisista monista elintarvikkeista oli myrkyllisyyden aiheuttaja ja siten ehkäistä lisäsairastumisia. Myrkyn tunnistaminen kereulidiksi tapahtuu massaspektrometrisesti. Osoitin että kun tämä tehdään käyttäen kereulidin molekyylijonien massalukuja: m/z (± 0.3) 1153.8 ($M+H^+$), 1171.0 ($M+NH_4^+$), 1176.0 ($M+Na^+$) ja 1191.7 ($M+K^+$), tunnistus on aukoton. Mikäli tuotetta ei säilytetä kylmässä ja myrkkyä tuottava bakteeri on läsnä niin mm. retkieväinä käytetyissä liha- ja karjalanpiirakoissa muodostuu yleisen myyntiajan puitteissa sairastumisen aiheuttavia määriä, 0.3–5.5 µg kereulidia grammassa elintarviketta.

Koska *Bacillus cereuksen* esiintyminen on niin yleistä, ettei siitä ole mahdollista päästä täysin eroon, on tärkeää tietää mitkä olosuhteet käynnistävät toksiinin tuoton. Tutkimuksissani selvisi että kereulidin tuotto voi vaihdella 10…1000 kertaisesti, olosuhteista riippuen. Kun elintarvike oli suljettuna astiaan, jonka kaasutila sisälsi vain typpikaasua (99.5 %), kereulidia ei muodostunut. Sen sijaan jos läsnä oli myös hiilidioksidia, kereulidia muodostui, vaikka happea oli vain alle 1 %. Myös lisä-aineilla oli vaikutusta kereulidin tuottoon, ainakin laboratorio-olosuhteissa. Leusiini ja valiini moninkertaistivat kereulidin tuoton. Peptidimuodossa nämä aminohapot ovat kaikkien proteiinien luontainen ainesosa. Yllättävää oli että vapaassa muodossa kasvatusalustaan lisätty leusiini ja valiini moninkertaistivat kereulidin tuoton, mutta proteiiniin sitoutuneilla aminohapoilla ei vastaavaa vaikutusta havaittu. Sekä leusiini että valiini ovat yleisesti käytettyjä valmisruokien aromivahventeita. Tutkimustulokseni osoittavat että nämä lisäaineet voivat aiheuttaa ruokamyrkytysriskin vaikkeivat itse ole lainkaan myrkyllisiä.

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9. References

Abriouel H. B., Omar N. L., Lopez R. M., Canamero M. Ortega E. Galvez A. 2007. Differentiation and characterization by molecular techniques of *Bacillus cereus* group isolates from poto poto and degue, two traditional cereal-based fermented foods of Burkina Faso and Republic of Congo. Journal of Food Protection 70: 1165-1173

Agata N., Mori M., Ohta M., Sathorn S., Ohtani I., and Isobe M. 1994. A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation In Hep-2 Cells. FEMS Microbiology Letters 121:31-34

Agata N., Ohta M., Mori M. and Isobe M. 1995. A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiology Letters 129:17-20

Agata N., Ohta M., and Mori M. 1996. Production of an emetic toxin, cereulide, is associated with a specific class of *Bacillus cereus*. Current Microbiology 33: 67-69

Agata N., M. Ohta, M. Mori, and K. Shibayama. 1999. Growth conditions of an emetic toxin production by *Bacillus cereus* in a defined medium with amino acids. Microbiology Immunology 43:15-18

Agata N., Ohta M., and Yokoyama K. 2002. Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. International Journal of Food Microbiology 73: 23-27

Andersson M. A., Mikkola R., Helin J., Andersson M. C., and Salkinoja-Salonen M. 1998. A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. Applied and Environmental Microbiology 64: 1338-1343

Andersson M., Mikkola R., Apetroaie C. Hoornstra D., Nieminen T. and Salkinoja-Salonen M. 2002. Fungicidic and mitochonriotoxic Bacilli frequent in water damaged buildings. In Proceedings of 9 th International Conference on Indoor Air Quality and Climate 2002 Vol 1: 34-39

Andersson M. A. Hakulinen P., Honkalampi-Hämäläinen U., Hoornstra D., Lhuquenot J. C., Mäki-Paakkanen J., Savolainen M., Severin I., Stammati A.-L., Turco L., Weber A.,von Wright A., Zucco F. and Salkinoja-Salonen M. 2007. Toxicological profile of cereulide, the *Bacillus cereus* emetic toxin, in functional assays with human, animal and bacterial cells. Toxicolon 49: 351-367

Altayar M. and Sutherland A. 2005. *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. Journal of Applied Microbiology 100: 7-14

AOAC, 1995, *Bacillus cereus* in foods. Enumeration and confirmation. Microbiological methods, AOAC official method 980:31, In Cunniff P. (Ed.), Official methods of analysis of AOAC International, 16th ed. Vol 1, AOAC International, Arlington VA, USA pp. 52-54

Apetroaie C. , Andersson M.A, Spröer C., Tsitko I., Shaheen R., Jääskeläinen E.L, Wijnands L.M, Heikkilä R. and Salkinoja-Salonen M. S. 2005. Cereulide producing strains of *Bacillus cereus* show diversity. Archives of Microbiology 184: 141-151

Beattie S. H. and Williams A. G. 2000. Detection of toxins. In Encyclopedia of Food Microbiology (vol 1). Edited by Robinson R. K., Batt C. A. and Patel P. D.Academic Press, San Diego, USA. pp: 141-149

Beecher H. and Wong A.C.L. 1994. Identification and analysis of the antigens detected by two commercial *Bacillus cereus* diarrheal enterotoxin immunoassay kits. Applied and Environmental Microbiology 60: 4614-4616

Carlin F., Fricker M., Pielaat A., Heisterkamp S., Shaheen R., Salkinoja-Salonen M., Svensson B., Nguyen-the C. and Ehling-Schulz M. 2006. Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group. International Journal of Food Microbiology 109: 132-138

Cronin U. P. and Wilkinson M. G. 2007. The use of flow cytometry to study the germination of *Bacillus cereus* endospores. Cytometry part A 71A: 143-153

Dierick R., van Coillie E., Swiecicka I., Meyfroidt H., Devlieger A., Meulemans A., Hoedemaekers G., Fourie L., Heyndrickx M. and Mahillon J. 2005. Fatal family outbreak of *Bacillus cereus*-associated food poisoning. Journal of Clinical Microbiology 43: 4277-4279

Ehling-Schulz M., Fricker M., Scherer S. 2004. Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Microbiology Letters 232: 189- 195

Ehling-Schulz M., Vukov N., Schulz A., Shaheen R., Andersson M. Martlbauer E. Scherer S. 2005a. Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. Applied & Environmental Microbiology 71:105-113

Ehling-Schulz M., Svensson B., Guinebretiere MH., Lindback T., Andersson M., Schulz A., Fricker M., Christiansson A., Granum PE., Martlbauer E., Nguyen-The C.,

Salkinoja-Salonen M. and Scherer S. 2005b. Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology 151:183-197

Ehling-Schulz M., Guinebretiere M-H., Monthan A., Berge O., Fricker M. and Svensson B. 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus.* FEMS Microbiology Letters 260: 232-240

European Commission, 1993. Council Directive 93/88/EEC modifying Directive 90/697/EEc on the protection of workers from risks related to exposure to biological agents to work. Official Journal of European Communion. L268 of 29.10.1993, pp: 71-82

European Commisson, 2006. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Acids and Materials in contact with Food (AFC) on request from the Commussion related to: Flavouring Group Evaluation 26: Amino acids from chemical group 34. Commission Regulation (EC) No 1565/2000 of July 2000. Adopted on 4.5.2006. The EFSA Journal 373: 1-48

Fagerlund A., Ween A., Lund T., Hardy S.P. and Granum P.E. 2004. Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. Microbiology 150: 2689-2697

Fagerlund A. Brillard J. Furst R. Guinebretiere MH. Granum PE. 2007. Toxin production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group. BMC Microbiology 7:43

Finlay W. J., Logan N.A., and Sutherland A.D. 1999. Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin. Applied and Environmental Microbiology 65:1811-1812

Finlay W.J., Logan N.A., Sutherland A.D. 2000. *Bacillus cereus* produces most emetic toxin at lower temperatures. Letters in Applied Microbiology 31: 385-389

Finlay W.J.J., Logan N.A. and Sutherland A.D. 2002. *Bacillus cereus* emetic toxin production in relation to dissolved oxygen tension and sporulation. Food Microbiology 19: 423-430

Food and Drug Administration (FDA), 1998. *Bacillus cereus*. In: Bacteriological Analytical Manual. 8th Edition, revision A, AOAC International, Gaithersburg, MD, USA, pp: 14.01-14.08

Forsythe S. J. 2000. Basic aspects. In: The microbiology of safe food. Blackwell Science. Edited by Forsythe S. MA, USA, pp: 10-52

Fricker M., Messelhäuβer, Busch U., Scherer S., Ehling-Schulz M. 2007. Diagnostic real-time PCR assay for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. Applied and Environmental Microbiology 73: 1892-1898

From C., Hormazābal V., Hardy S.P. and Granum P.E. 2007. Cytotoxicity in *Bacillus mojavensis* is abolished following loss of surfactin synthesis: Implications for assessment of toxicity and food poisoning potential. International Journal of Food Microbiology 117: 43-49

Granum P. E. 2002. *Bacillus cereus* and food poisoning. In Applications and systematics of Bacillus and relatives, edited by Berkeley R., Heyndrickx M., Logan N. and De Vos P. Blackwell publishing company. MA, USA. pp: 37-46

Granum P.E. 2007. *Bacillus cereus*. In: Food Microbiology: Fundaments and Frontiers, 3 rd ED. Edited by Doyle M. and Beuchat L. 3rd Edition, ASM Press, Washington, D.C. pp: 445-456

Griffiths M. W. and Schraft H. 2002. *Bacillus cereus* food poisoning. In Foodborne diseases, edited by Clicer D. O. and Riemann H. P. Academic press, California USA, pp: 261-270

Guinebretière M-H., Broussolle V. and Nguyen-The N. 2002. Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. Journal of Clinical Microbiology 40: 3053-3056

Guinebretière M-H. Fagerlund A. Granum PE. and Nguyen-The C. 2006. Rapid discrimination of cytK-1 and cytK-2 genes in *Bacillus cereus* strains by a novel duplex PCR system. FEMS Microbiology Letters 259: 74-80

Han, C.S., Xie, G., Challacombe, J.F., Altherr, M.R., Bhotika, S.S., Brown, N., Bruce, D., Campbell, C.S., Campbell, M.L., Chen, J., Chertkov, O., Cleland, C., Dimitrijevic, M., Doggett, N.A., Fawcett, J.J., Glavina, T., Goodwin,L.A., Green, L.D., Hill, K.K., Hitchcock, P., Jackson, P.J., Keim, P., Kewalramani, A.R., Longmire, J., Lucas, S., Malfatti, S., McMurry, K., Meincke, L.J., Misra, M., Moseman, B.L., Mundt, M., Munk, A.C., Okinaka,R.T., Parson-Quintana,B., Reilly, L.P., Richardson, P., Robinson, D.L., Rubin,E., Saunders, E., Tapia, R., Tesmer, J.G., Thayer, N., Thompson ,L.S., Tice, H., Ticknor, L.O., Wills, P.L., Brettin, T.S. and Gilna, P. 2006. Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. Journal of Bacteriology 88: 3382-3390

Hallaksela A.-M., Väisänen O. and Salkinoja-Salonen M. 1991. Identification of Bacillus species isolated from *Picea abies* by physiological tests, phage typing and fatty acid analysis. Scandinavian Journal of Forest Research 6: 365-377.

Henriques A. O. and Moran C. P. 2007. Structure, assembly, and function of the spore surface layers. Annual Reviews of Microbiology 61: 555-588.

Helgason, E., Økstad, O.A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. and KolstØ A. B. 2000. *Bacillus anthracis, Bacillus cereus* and *Bacillus thuringiensis* - one species on the basis genetic evidence. Applied and Environmental Microbiology 66: 2627-2630.

Holt J.G., Frieg N.R, Sneath P.H.A Staley S.T. and Williams S.T. 1994. Genus Bacillus. In Bergey´s manual: Determinative bacteriology 9 th edition. Edited by Hensyl W.R. Baltimore, USA Williams & Wilkins. pp: 559-564

Hoornstra D., Andersson M.A., Mikkola R. and Salkinoja-Salonen M.S. 2003. A new method for in vitro detection of microbially produced mitochondrial toxins. Toxicology in Vitro 17: 745-751

Hoornstra D., Dahlman O., Jääskeläinen E., Andersson M., Weber A., Aurela B., Lindell H. and Salkinoja-Salonen M., 2006. Retention of *Bacillus cereus* and its toxin, cereulide, in cellulosic fibres. Holzforschung 60: 648-652

Hormazābal V., ∅yvin ∅., O´Sullivan K. and Granum P.E. 2004. Quantification of *Bacillus cereus* emetic toxin (cereulide) in figs using LC/MS. Journal of Liquid Chromatography and Related Techniques 27: 2531-2538.

Hornstra L. M., de Vries Y. P, de Vos W. M., Abee T. 2006. Influence of sporulation medium composition on transcription of *ger* operons and the germination response of spores of *Bacillus cereus* ATCC 14579. Applied and Environmental Microbiology 72: 3746-3749.

Hornstra L.M, de Leeuw P.L., Moezelaar R., Wolbert E.J., de Vries Y.P., de Vos W.M. and Abee T. 2007. Germination of *Bacillus cereus* spores adhered to stainless steel. International Journal of Food Microbiology 116: 367-371

Horwood PF., Burgess GW. Oakey HJ. 2004. Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. FEMS Microbiology Letters 236:319-24

Hoton FM., Andrup L., Swiecicka I., and Mahillon J. 2005 The cereulide genetic determinants of emetic Bacillus cereus are plasmid-borne. Microbiology 151: 2121-2124

Hughes S., Bartholomew B., Hardy J.C. and Kramer J.M. 1988. Potential application of a HEp-2 cell assay in the investigation of *Bacillus cereus* emetic-syndrome food poisoning. FEMS Microbiology Letters 52:7-11

Häggblom M. M., Apetroaie C., Andersson M. A., and Salkinoja-Salonen M. 2002**.** Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus,* produced under different conditions. Applied and Environmental Microbiology 68 : 2479-2483

IDF (International Dairy Federation) 1998. Dried Milk Products. Enumeration of *Bacillus cereus.* Most probable number technique. Standard method 181.1998 International Dairy Federation, Brussels, Belgium.

ISO (International Organisation for Standardisation), 2004. Microbiology of food and animal feeding stuffs - horizontal method for the enumeration of presumptive *Bacillus cereus*-colony-count technique at 30°C. 3rd edition, Standard 7932:2004 Geneve, Switzerland

Ivanova N., Sorokin A., Endersin I., Galleron N., Candelon B., Kapatral V., Bhattacharyya A., Reznik G., Mikhailova N., Lapidus A., D´Souza M., Walunas T., Grechkin Y., Pusch G., Haselkorn R., Fonstein M., Ehrlich S. D., Overbeek R. and Kyprides N. 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature 423: 87-91

Jay, J.M., Loessner M.J., Golden D.A. 2005. *Bacillus cereus* gastroenteritis. In: Modern food microbiology, 7th edition, Springer Science+Business media, Inc., New York, USA pp: 583-590

Jensen G.B., Hansen B.M., Eilenberg J and Mahillon J. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Minireview in Environmental Microbiology 5: 631-640

Kawamura-Sato K., Hirama Y., Agata N., Ito H., Torii K., Takeno A., Hasegawa T., Shinomura Y., and Ohta M. 2005. Quantative analysis of cereulide, an emetic toxin of *Bacillus cereus*. Microbiology Immunology 49:25-30

Kolst∅ A.-B, Lereclus D. and Mock M. 2002. Genome structure and evolution of the *Bacillus cereus* group. Current Topics in Microbiology and Immunology 264:95-108

Kramer J.M. and Gilbert R.J. 1989. *Bacillus cereus* and other *Bacillus* species. In Foodborne Bacterial Pathogens. ed. Doyle, M.P. Marcel Decker, New York, pp: 22-70

Lund T. and Granum P.E. 1996. Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiology Letters 141: 151-156

Lund T., De Buyser ML and Granum PE. 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Molecular Microbiology 38: 254-261

Mahler H., Pasi A., Kramer J., Schulte P., Scoging A., Baer W., and Kraehenbuehl S. 1997. Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. New England Journal of Medicine 336:1143-1148

Mikami, T., Horikawa, T., Murakami, T., Matsumoto, T., Yamakawa, A., Murayama, S. Katagiri, S., Shinagawa, K., Suzuki, M. 1994. An improved method for detecting cytostatic toxin (emetic toxin) of *Bacillus cereus* and its application to food samples. FEMS Microbiology Letters 119: 53-58

Mikkola R., Saris N.E., Grigoriev P.A., Andersson M.A. and Salkinoja-Salonen M.S. 1999. Ionophoretic properties and mitochondrial effects of cereulide: the emetic toxin of *B. cereus*. European Journal of Biochemistry 263:112-117.

Moravek M., Buerk C., Broussolle V., Guinebretiere M-H., Granum P.E., Nguyen-The C. and Märtlbauer E. 2006. Determination of the toxin potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. FEMS Microbiology letters 257: 293-298.

Mortimer P.R. and McCann G. 1974. Food-poisoning episodes associated with *Bacillus cereus* in fried rice. Lancet. 1(7865):1043-1045.

NCBI: http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=search&term=bacillus+cereus. 31.10.2007

Nishikawa Y., Kramer J. M, Hanaoka M. and Yasukawa A. 1996. Evaluation of serotyping, biotyping, plasmid banding pattern analysis, and HEp-2 vacuolation factor assay in the epidemiological investigation of *Bacillus cereus* emetic-syndrome food poisoning. International Journal of Food Microbiology 31: 149-159

Niskanen T, Johansson T., Kuusi M., Raahenmaa M, Siitonen A., Tuominen P. 2006 Ruokamyrkytykset Suomessa vuonna 2005. Eviran julkaisuja 2/2006 sivut: 17-27

Niskanen T., Johansson T., Siitonen A. and Kuusi M. 2007. Ruokamyrkytykset Suomesa vuonna 2006. Eviran julkaisuja 21/2007 sivut: 15-28

Nordic Committee on Food Analysis (NMKL). 1997. *Bacillus cereus*. Determination in foods. UDC 579.852.11. Method No. 67, 4th ed. Oslo, Norway

Paananen A., Mikkola R., Sareneva T., Matikainen S., Hess M., Andersson M., Julkunen I., Salkinoja-Salonen M.S. and Timonen T. 2002. Inhibition of human natural killer cell activity by cereulide, an emetic toxin from *Bacillus cereus*. Clinical and Experimental Immunology 129: 420-428

Parry J. M., Turnbull P.C.B and Gibson J.R. 1983. A color atlas of *Bacillus* species, Wolfe Mecical Publications Ltd, London, UK, pp: 98-107

Pirhonen T., Andersson M., Jääskeläinen E., Salkinoja-Salonen M. Honkanen-Buzalski T., Johansson T. 2005. Biochemical and toxic diversity of *Bacillus cereus* in a pasta and meat dish associated with a food poisoning. Food Microbiology 22: 87-91

Pirttijärvi, T. S., Andersson M.A., Scoging A.C., and Salkinoja-Salonen M.S. 1999. Evaluation of methods for recognizing strains of the *Bacillus cereus* group with food poisoning potential among industrial and environmental contaminants. Systematic & Applied Microbiology. 22: 133-144

Pirttijärvi T. 2000. Contaminant aerobic sporeforming bacteria in the manufacturing processes of food packaging board and food. Dissertationes Biocentri Viikki Universitatis Helsingiensis 14/2000. Ph.D. thesis, University of Helsinki, Finland

Pirttijärvi T.S.M., Andersson M.A. and Salkinoja-Salonen M.S. 2000. Properties of *Bacillus cereus* and other bacilli contaminating biomaterial-based industrial processes. International Journal of Food Microbiology 60: 231-239

Rajkovic A., Uyttendaele M., Deley W., Van Soom A., Rijsselaere T. and Debevere J. 2006a. Dynamics of boar semen motility inhibition as a semi-quantitative measurement

of *Bacillus cereus* emetic toxin (cereulide). Journal of Microbiological Methods 65: 525-534

Rajkovic A., Uyttendaele M., Ombregt S.-A., Jääskeläinen E., Salkinoja-Salonen M. and Debevere J. 2006b. Influence of type of food on the kinetics and overall production of *Bacillus cereus* emetic toxin. Journal of Food Protection 69: 847-852

Rasko D.A., Ravel J., ∅kstad O.A., Helgason E., Cer R.Z., Jiang L., Shores K.A., Fouts D.E., Tourasse N.J., Angiuoli S.V., Kolonay J., Nelson W.C., Kolst∅ A.B., Fraser C.M and Read T.D. 2004. The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. Nucleic Acids Res 32: 977-988

Rasko D.A., Rosovitz M.J,. Okstad O.A,. Fouts D.E., Jiang L., Cer R.Z., Kolsto A.B., Gill S.R. and Ravel J. 2007. Complete sequence analysis of novel plasmids from emetic and periodontal *Bacillus cereus* isolates reveals a common evolutionary history among the *B. cereus*-group plasmids, including *Bacillus anthracis* pXO1. Journal of Bacteriology 189:52-64

Reers M., Smiley S.T., Mottola-Hartshorn C., Chen A., Lin M. and Chen L. B. 1995. Mitochondrial membrane potential monitored by JC.1. Methods in Enzymology 260: 406-417

Roberts T.A., Baird-Parker A. C. and Tompkin R. B. 1996. *Bacillus cereus*. In: Micro-organisms in Foods. 5. Microbiological Specification of Food Pathogens. Blackie Academic & Professional 1996 Great Britain. pp: 20-35

Rosenquist H., Smidt L., Andersen S., Jensen G. and Andrea Wilcks. 2005. Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. FEMS Microbiology Letters 250: 129-136

Sagripanti J-L., Carrera M., Insalaco J., Ziemski M., Rogers J. and Zandomeni R. 2006. Virulent spores of *Bacillus anthracis* and other *Bacillus* species deposited on solid surfaces have similar sensitivity to chemical decontaminants. Journal of Applied Microbiology 102: 11-21

Sakurai N., Koike, K.A., Rie, Y. & Hayashi, H. 1994. The rice culture filtrate of *Bacillus cereus* isolated from emetic-type food poisoning causes mithochondrial swelling in a HEp-2 Cell. Microbiology Immunology 38: 277-343

Seibert H., Mörcher S., Gulden M. 2002. Factors influencing norminal effective concentrations of chemical compounds in vitro: medium protein concentration. Toxicology In Vitro 16: 289-297

Shaheen R., Andersson M.A., Apetroaie C., Schulz A., Ehling-Schulz M., Ollilainen V-M. and Salkinoja-Salonen M.S. 2006. Potential of selected infant food formulas for production of *Bacillus cereus* emetic toxin, cereulide. International Journal of Food Microbiology 107: 287-294

Shinagawa, K., 1993. Serology and characterization of toxigenic *Bacillus cereus*. Netherland Milk Dairy J., 47, 89-103

Shinagawa K., Konuma H., Sekita H. and Sugii S. 1995. Emesis of rhesus monkeys induced by intragastric administration with the HEp-2 vacuolation factor, cereulide, produced by *Bacillus cereus*. FEMS Microbiology Letters 130: 87-90

Shinagawa K., Ueno Y., Hu D., Ueda S. and Sugii S. 1996. Mouse lethal activity of a HEp-2 vacuolation factor, cereulide, produced by *Bacillus cereus* isolated from vomiting type food poisoning. Journal of Veterinary Medical Sciences 58: 1027-1029

Sim R.B. 1998. *Bacillus cereus* gastroenteritis. In. Hausler W. and M. Sussman (eds.), Topley & Wilson's Microbiology and Microbial Infections, vol.3. Arnold, London, Great Britain pp: 551-556

Svensson B., Monthan A., Shaheen R., Andersson M. A., Salkinoja-Salonen M and Christiansson A. 2006. Occurrence of emetic toxin producing *Bacillus cereu*s in the dairy production chain. International Dairy Journal 16: 740-749

Swiecicka I. and Mahillon J. 2006. Diversity of commensal *Bacillus cereus sensu lato* isolated from the common sow bug (*Porcellio scaber*, Isopoda). FEMS Microbiology Letters 56: 132-140

Swiecicka I., Van der Auwera G. and Mahillon J. 2006. Hemolytic and nonhemolytic enterotoxin genes are broadly distributed among *Bacillus thuringiensis* isolated from wild animals. Microbial Ecology 52: 544-551

Szabo R. A. Speirs J. I. and Akhtar M. 1991. Cell culture detection and conditions for production of a *Bacillus cereus* heat-stable toxin. Journal of Food Protection 54: 272-276

Taylor A.J. and Gilbert R.J. 1975. *Bacillus cereus* food poisoning: a provional serotyping scheme. Journal of Medical Microbiology 8: 543-550

Teplova V., Jääskeläinen E., Salkinoja-Salonen M., Saris N.E., Serlachius M., Li F.Y. and Andersson L.C. 2004. Differentiated Paju cells have increased resistance to toxic effects of potassium ionophores. Acta Biochimica Polonica 51: 539-544

Teplova V.V., Mikkola R., Tonshin A. A., Saris N.-E.L. and Salkinoja-Salonen M.S. 2006. The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for Potassium at physiological plasma concentration. Toxicology Applied of Pharmacology 201:39-46

Thorsen L., Hansen B.M., Nielsen K.F., Hendriksen N.B,. Phipps R.K., and Budde B.B. 2006. Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium. Applied & Environmental Microbiology 72 :5118-21

Turnbull P. C. B., Kramer J.M., Jörgensen K., Gilbert R.J, and Melling J. 1979. Properties and production characteristics of vomiting, diarrhea and necrotizing toxins of *Bacillus cereus*. The American Journal of Clinical Nutrition. 32: 219-228

van Der Zwet W.C., Parlevliet G.A., Savelkoul P.H., Stoof J., Kaiser A.M., Van Furth A.M, and Vandenbroucke-Grauls C.M. 2000. Outbreak of *Bacillus cereus* infections in a neonatal intensive care unit traced to balloons used in manual ventilation. Journal of Clinical Microbiology 38: 4131-4136

Varnam A.H. and Evans M.G. 1991. Food poisoning: medical and microbiological overview; Bacillus. In foodborne pathogens, an illustrated text. Mosby year book, London, UK, pp: 9-19; 267-288

Vassileva M., Torii K., Oshimoto M., Okamoto A., Agata N., Yamada K., Hasegawa T. and Ohta M. 2007. A new phylogenetic cluster of cereulide-producing *Bacillus cereus* strains Journal of Clinical Microbiology 45: 1274-1277

Wang GYS, Kuramoto M., Yamada K., Yazakawa K. and Uemura D. 1995. Homocereulide, an extremely potent cytotoxic depsipeptide from the marine bacterieum *Bacillus cereus*. Chemistry Letters 9: 791-792

Warren S. C. and Gould G. W. 1968. *Bacillus cereus* spore germination: absolute requirement for an amino acid. Biochemica et Biophysica acta. 170: 341-350

Wijnands L. M., Dufrenne J. B., Rombouts F. M., in´t Veld P. H. and Leusden F. M. 2006. Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the Netherlands. Journal of Food Protection 69: 2587-2597

Wijnands L. M., Dufrenne J.B., van Leusden F.M and Abee T. 2007. Germination of *Bacillus cereus* spores is induced by germinants from differentiated Caco-2 cells, a human cell line mimicking the epithelial cells of the small intestine. Applied and Environmental Microbiology 73: 5052-5054

Yokoyama K., Ito M., Agata N., Isobe M., Shibayama K., Horii T. and Ohta M. 1999. Pathological effect of synthetic cereulide, an emetic toxin of *Bacillus cereus*, is reversible in mice. FEMS Immunology Microbiology 24: 115-120

Yousten A.A. 1975. Germination of *Bacillus cereus* endospores: a proposed role for heat shock and nucleosides (review). Canadan Journal of Microbiology 21: 1192-1197